EPIDEMIOLOGY OF TUBERCULOSIS IN CATTLE AND HUMAN PATIENTS IN BORNO AND YOBE STATES, NIGERIA

BY

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BY

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DEPARTMENT OF VETERINARY MEDICINE
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MARCH, 2016

DECLARATION

I hereby declare the originality of this Thesis entitled **''EPIDEMIOLOGY OF TUBERCULOSIS IN CATTLE AND HUMAN PATIENTS IN BORNO AND YOBE STATES, NIGERIA''** carried out by me in the Department of Veterinary Medicine, Ahmadu Bello University, Zaria under the supervision of Professors Idris A. Abdulkadir, J. P. Kwaga, H.G. Abdulrazak, and Lovett Lawson. The information derived from the literature has been duly acknowledged in the text and a list of reference provided. No part of this thesis was previously presented for another degree or diploma at this or any other Institution.

Bello Abubakar USMAN …….. ……...

Signature **Date**

CERTIFICATION

This thesis entitled **EPIDEMIOLOGY OF TUBERCULOSIS IN CATTLE AND HUMAN PATIENTS IN BORNO AND YOBE STATES, NIGERIA** by Bello Abubakar USMAN meets the regulations governing the award of the degree of Doctor of Philosophy in Veterinary Medicine of the Ahmadu Bello University, Zaria and is approved for its contribution to scientific knowledge and literary presentation.

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DEDICATION

This Thesis is dedicated to my parents, my late grandparents and my lovely family.

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ABSTRACT

Bovine tuberculosis has been on increasing concern and a threat to public health, especially in developing countries like Nigeria. This study was conducted in Borno and Yobe States, located in North Eastern Nigeria to study the epidemiology of bovine tuberculosis in cattle and human TB patients. Six diagnostic procedures namely, tuberculin testing, post-mortem inspection for TB-like lesions, culture, acid-fast staining, TB Ag MPT64 (SD-bioline) and genotype MTBC were used. Prevalence of bovine tuberculosis based on tuberculin test survey involving 109 cattle herds from 10 Zonal Veterinary Areas (testing areas) in the study area was described. A total of 6, 293 cattle were tested out of which 645 were positive with a prevalence of 10.3%. Prevalence of bovine tuberculosis based on gross TB-like lesions was determined. A total of 4,130 slaughtered cattle were examined and out of which 379 were compatible with gross TB-like lesions with a prevalence of 9.2%. This finding indicates that there is a relationship between the prevalence of bovine TB among slaughtered cattle and among live cattle using the tuberculin test. The results of the risk factors related to cattle handlers' awareness/knowledge of bovine TB indicated that males had higher percentage (87.3%) compared to females (48.3%), respondents above 40 years had higher percentage (82.9%) compared to those less than 40 years (78.4%). Level of formal education; tertiary level of education were more informed about bovine TB (96.6%) followed by those with secondary, primary and no formal education respectively (94.1%, 88.7% and 46.3%). Occupation; meat inspectors (100%) and butchers (98.1%) were more informed than cattle rearers, cattle marketers and milk sellers with 71.6%, 70.8% and 29.4% respectively. The results of the risk factors related to cattle handlers on the habit of drinking raw milk indicated that females had higher percentage (80.2%) compared to males (37.8%).

Based on age; respondents that were above 40 years had higher percentage (52.9%) compared to those that were less than 40 years (41.8%). Level of formal education; it was found that those who had no formal education had higher percentage (61.8%) followed by those with primary, secondary and tertiary with 55.1%, 54.8% and 22.4% respectively. Occupation of the respondents; It was higher among cattle rearers (80.2%) followed by milk sellers, cattle marketers, meat inspectors and butchers with 69.6%, 40.2%, 12.7% and 10.9%, respectively. Isolation and identification of tubercle bacilli from human sputa based on culture, acid- fast staining and TB Ag MPT64 (SD-bioline) procedures revealed 94(24.0%), 67(71.3%) and 62 (66.0%) isolates respectively. Out of the 67 isolates tested by Genotype MTBC molecular assay, 15(23.8%) were identified as *M. bovis*, 6(9.5%) were identified as *M. africanum* and 42(66.7%) were identified as *M. tuberculosis*. However, it is worth noting that four *M. bovis* isolates were traced to human while the remaining 11 were traced to cattle. One *M. africanum* isolate was traced to cattle while the remaining 5 were traced to human. Two *M. tuberculosis* isolates were traced to cattle while the remaining 40 were traced to human; thus indicating human-to-animal and animal-to-human transmissions. The study highlighted the importance of tuberculosis in cattle and humans and its public health implications and calls for prompt action towards controlling the disease in the two States and Nigeria in general.

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LIST OF ABBRIVIATIONS

AFS……Acid-Fast Stain

bTB …… Bovine Tuberculosis

BCG…… Bacillus Calmette–Guérin

CDC…… Centre for Disease Control and Prevention

CFU…… .Colony Forming Unit

CFP-10…… .Culture Filtrate Protein-10

CMI…… .Cell-mediated Immunity

DOTS……Directly Observed Therapy-Short Course Strategy

DR…… Direct repeat region

DTH…… Delayed Type Hypersensitivity

ELISA…… .Enzyme-Linked Immuno-Sorbent Assay

EPI…… Expanded Programme on Immunization

ESAT-6…… Early Secretary Antigenic Target-6

GTBL……Gross-Tuberculosis Lesions

IFN-y……Gamma Interferon

IL…… .Interleukin

INH……Isoniazid

LBA……Lymphocyte Blastogenic Assays

M.bovis……*Mycobacterium bovis*

MAPIA……...Multi Agent Print Immunoassay

MPTR…… .Major polymorphic tandem repeats

MIRUs……Mycobacterial interspersed repetitive units

MTBC……*Mycobacterium tuberculosis* Complex

NPI……National Programme of Immunizations

NTBCP……National TB and Leprosy Control Programme

OIE…… Office of the International Epizootics

PCR……Polymerase Chain Reaction

PM……...Post- mortem

PPD……Purified Protein Derivative

PFGE……..Pulse Field Gel Electrophoresis

PGRS……Polymorphic Guanine and Cytosine-rich Repetitive Sequences

PZA…… Pyrazinamide

RFLP……Restriction Fragment Length Polymorphism

REA…… Restriction Endonuclease Analysis

SIDT…… Single Intradermal Tuberculin

TB…… Tuberculosis

TNF……Tumor Necrotic Factor

UN……..United Nations

USAID……..United States Agency for International Development

VNTR……Variable Number of Tandem Repeats

WHO…….World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Tuberculosis (TB) is a chronic infectious and contagious disease of domestic animals, wild animals and humans (Radostits *et al.,* 2010). It is characterized by the formation of granulomas in tissues especially in the lungs, lymph nodes, intestines, liver and kidneys (Shitaye *et al.,* 2007). It is caused by pathogenic members of the genus *Mycobacterium* which are commonly known as members of *Mycobacterium tuberculosis* complex (*Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium africanum, Mycobacterium microti and Mycobacterium cannetti)* (Collins and Grange, 1983; Pfeiffer, 2003).

Tuberculosis is widely distributed throughout the world with serious effect on animals and is also of significant public health importance (O'Reilly and Daborn, 1995). The disease in humans is known by several common names such as wasting disease, consumption, white plague and pearl disease (Abubakar, 2007).

Nigeria has a population of over 140 million people (National Population Commission, 2006) and ranks 10th among the world's 22 countries with a high TB burden (WHO, 2005a). Among African countries, Nigeria has the highest incidence TB cases with nearly 368,000 new cases and an estimated 30,000 deaths annually (WHO, 2005b).

As in most developing countries, in Nigeria, Bacillus Calmete-Guerin is the first vaccine given in the routine vaccination schedule of the National Programme of Immunization (NPI) and most children receive BCG at birth or as soon as possible after birth. The programme was first initiated in 1979 when it was known as the Expanded Programme on Immunization (EPI) (Odusanya *et al.,* 2003). The funds for acquisition of vaccines were sourced from both

the government and donor agencies such as the World Health Organization (WHO) and other charitable organizations (Abubakar, 2007).

The Nigerian government formally launched its National TB and Leprosy Control Programme (NTBLCP) in 1991 and adopted the WHO recommended 'Directly Observed Therapy-Short Course Strategy' (DOTS) in 1993. The DOTS programme was established as the control method for tuberculosis in humans and integrated into the Primary Health Care of the country through the Federal Ministry of Health (Wim *et al.,* 2004).

Despite this expression of political will to control human TB in Nigeria, and a clearly articulated national TB policy, there was no sufficient financial support necessary for the effective implementation of the policy (Anon, 2006). Effective TB control is also affected by inadequate trained personnel and political instability, poor health infrastructure and facilities, poor referral system, poor procurement and supply management system and poor donor coordination/management locally and internationally. Most importantly also, diagnosis of tuberculosis stops at the smear level, hence the species involved in causing the disease are not known thereby making it difficult to study outbreaks, trace the routes of transmission and also identify the species and strains of *Mycobacterium* involved. Another major setback is the inability of the national TB control program to recognize the significance of *M. bovis*, which are a major public health problem and the general lack of collaboration between human and veterinary medical personnel in this regard. Generally, there has been total lack of synergy and harmony of laws regulating the control of human and bovine tuberculosis in the country (Abubakar, 2010). The TB program in Nigeria is donor-dependent with United Nation (UN) and Global Fund as the major donors. UN support includes United States Agency for International Development (USAID), Centre for Disease Control and Prevention (CDC) and DODS as implementing partners. Other development partners include: WHO; Leprosy Mission in Nigeria; Netherland Leprosy Relief; German Leprosy and TB Relief Assocition;

Damien Fundation of Belgium; International Union against TB and Lung Diseases; Canadian International Development Agency; and UK Department for International Development (DFID) (Gambo *et al.*, 2013).

Nigeria has an estimated cattle population of over 20 million, with the Zebu breed constituting over 90% of the total national herd (Ariyo, 2002). The first report of the existence of *Mycobacterium bovis* in West Africa as a causative agent of bovine tuberculosis was in 1913 by Ziemann in Cameroon (Alhaji, 1976). But the first report of the existence of bovine tuberculosis in Nigeria based on tuberculin test results and then followed by post mortem and laboratory examination was in 1929, cited by (Alhaji, 1976). Other studies have further confirmed the existence of bovine tuberculosis in the west African region and Nigeria in particular (Alhaji, 1976; Eid, 1976; Ayanwale, 1984; Abubakar, 1994; Du-Sai and Abdullahi, 1994; Ankugah, 2002; Cadmus *et al.*, 2004; Awah-Ndukum *et al.*, 2005; Abubakar, 2007; Abubakar, 2010). Some other studies in humans have also shown the involvement of *M. bovis* in causing tuberculosis, for instance, in a study of 102 *M. tuberculosis* complex from patients with pulmonary TB, 4 (3.9%) were found to be *M. bovis* (Idigbe *et al.*, 1986). Cadmus and colleagues (2006) have also isolated 3 *M. bovis* strains in a study of 55 human sputum samples in Ibadan, Nigeria.

The control of bTB in Nigeria is regulated by the Federal Ministry of Agriculture. However, this control policy as stated in the Animal Diseases (Control) Decree of 1988 is poorly or inadequately implemented in recent years (Federal Republic of Nigeria, 1988). This is largely due to politico-economic reasons, such as the high cost of sustainable testing and slaughter of infected animals and the subsequent compensation to farmers. Added to this is the problem of social unrest due to political instability and ethnic wars especially between the Nomadic

Fulani herders and local farmers, resulting in the displacement of large numbers of human and animal populations (Ayele *et al.*, 2004). Also, socially, the test and slaughter method of controlling bTB is not accepted by the Fulani nomads who own the largest cattle population in Nigeria. There is also the lack of adequate veterinary personnel to implement control measures such as rigorous meat inspection at abattoirs. Poor communication networks, insufficient collaboration with neighbouring countries and the lack of quarantine, and smuggling of live animals across state boundaries have also been identified to cause problem in controlling the disease in cattle (Ayele *et al.*, 2004).

1.2 Statement of the Research Problems

In 1993 the WHO declared TB a 'global emergency' (WHO 2002a), the first to be declared as such. More than a decade after such declaration, the disease still remained one of the world's leading causes of death among adults (WHO 2003a). More than 8 million people developed active TB annually, and approximately 2-3 million died from the disease each year (WHO, 2003a). More than 80% of all TB patients live in sub-Saharan Africa and Asia (WHO, 2006). It has been estimated that, 200 million people are at risk of developing the disease between year 2001 to 2021 (van Soolingen, 2001).

Tuberculosis is also recognized worldwide as a significant animal health risk, primarily in domestic cattle. Despite considerable success in controlling this disease in cattle populations in many developed countries, tuberculosis remains an important economic and health problem in others, especially Africa, mostly due to socio-political reasons (Morris, 1994).

Bovine TB is a zoonotic disease with potential public health and socio-economic significance (Ayele *et al.*, 2004). However, unlike in the developed regions of the world, the extent of

involvement of *Mycobacterium bovis* in human tuberculosis is not well known and its impact on the high-risk group with concomitant HIV/AIDs infection in the developing countries is a growing concern (Neill *et al.*,2005). This is a complication in the recent global resurgence of human tuberculosis, which has precipitated renewed research interest.

From the public health point of view and based on the reports of the prevalence of both human and bovine tuberculosis in Nigeria, it can be concluded that *Mycobacterium bovis* is prevalent in Nigeria and indeed, some percentage of the public stand the risk of getting infected. Also the information on specie of *Mycobacterium* involved in causing the disease in both humans and animals in Nigeria is scanty. All these need further investigation and are to be made a research priority if effective control of TB is to be achieved. Specie identification of Mycobacterium responsible in causing the disease has been used for epidemiological investigation of tuberculosis and also for identification of potential sources of infection both in the general population and the nosocomial settings to help in developing better control measures. Molecular techniques now valuable tool in achieving this (Abubakar, 2007).

1.3 Justification of the Study

In Nigeria, information on the epidemiology and public health significance of *M. bovis* infection is very scanty. The only available information is mostly on limited surveys carried out on individual basis and scanty records from abattoirs (Cadmus *et al.,* 2004). These disjointed studies, make understanding of the magnitude of the problem difficult. The epidemiology and public health significance of bovine tuberculosis in Nigeria remains largely unknown. What is known about bovine tuberculosis (bTB) mostly comes from the granulomatous lesions found at slaughter houses/abattoirs and extremely sporadic tuberculin tests conducted in some states and few privately owned herds (Abubakar, 2007).

There is also failure or inadequate implementation of control policies for bovine tuberculosis, such as whole herd tuberculin testing, slaughter and rigorous meat inspection to control and monitor epidemiology of the disease. This is largely due to politico-economic reasons, such as the high cost of sustainable testing and a compensation program, inadequate trained veterinary personnel and the occasional political instability in the country.

On the aspect of human tuberculosis, as a routine, identification of the organism stops mostly at the direct smear examination level in most Government owned hospitals. All these lead to general under estimation of the importance of zoonotic TB in the public health sector at all the three tiers of government (Abubakar, 2007).

The use of molecular techniques for epidemiological studies has not been fully exploited in Nigeria; as such there is limited data on the strains of *Mycobacterium* responsible for both human and bovine tuberculosis (Cadmus *et al*., 2006).

These problems make epidemiological studies possible only on the basis of the limited field survey readily carried out and the scanty records of cases from abattoirs and hospitals. Generally, there is lack of synergy and harmony of laws and regulations between veterinary and medical policies for the control of tuberculosis in Nigeria. In view of this, it is therefore justified that this study; Epidemiology of Bovine tuberculosis in Cattle and Humans in Borno and Yobe States, Nigeria be embarked upon.

1.4 Aim of the Study

The aim was to carry out a study on the Epidemiology of bovine tuberculosis in cattle and humans, in order to improve the level of information on bovine TB in Borno and Yobe states, Nigeria.

1.5 Objectives of the Study

Based on the problems stated and research questions raised, the specific objectives are

1. To determine the prevalence of bovine tuberculosis infection in cattle, based on tuberculin testing, and gross TB-like lesions in slaughtered cattle in Borno and Yobe States.
2. To isolate species of *Mycobacterium* from cattle and human samples, through culture and Acid-fast staining,
3. To identify species of *Mycobacterium* from cattle and human isolates, through TB Ag MPT64 (SD-bioline) and Genotype MTBC Assay (Molecular biology techniques)
4. To assess risk factors associated with bovine tuberculosis through application of questionnaire to animal handlers.

1.6 Research Questions

1. What is the prevalence of bovine TB in cattle in Borno and Yobe States
2. What are the species of Mycobacterium responsible for TB in cattle and humans in Borno and Yobe States
3. What are the risk factors associated with bovine TB in Borno and Yobe States

CHAPTER TWO

LITERATURE REVIEW

2.1 Historical Background

The history of tuberculosis alerts one to put into perspective, man's struggle against the "White plaque" which has caused and is still causing a mighty burden of illness and death of humans and animals (WHO, 2005a). Tuberculosis has affected humans and animals since ancient times. The disease was described in Italian writing more than 2000 years before Christ was born (Salo *et al.*, 2003).

Tuberculosis (TB) in animals, which was also called "Pearl-disease" attracted attention thousand years ago and the early meat inspection regulations in various countries were concerned with this form of the disease (Salo *et al.*, 1994). The danger of eating meat from tuberculosis-infected animal existed in Mosaic laws and the German regulations banning the sale of tuberculous meat (Collins and Grange, 1983).

Generally, the turning point in the history of the genus *Mycobacterium* and tuberculosis in particular, occurred in 1852 when a German scientist, Robert Koch, publicly announced that he had observed and cultured the bacillus responsible for tuberculosis (Groschel, 1982).

The theory of infectiousness of tuberculosis was placed upon firm foundation and research work since then. This has been directed towards understanding the epidemiology of the disease and the means by which it can be controlled and eventually eradicated (Alhaji, 1976).

2.2 Aetiology

It is thought that the progeny of the *Mycobacterium tuberculosis* complex, comprising *Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium africanum, Mycobacterium microti and Mycobacterium cannetti*, arose from a soil bacterium and that the human bacillus may have been derived from the bovine form following the domestication of cattle (Collins and Grange, 1983). The aetiologic agent of bovine tuberculosis is primarily *Mycobacterium bovis*. It was first clearly differentiated from other type of tubercle bacilli by Theobald Smith (1898). It has a much wider range of pathogenecity for defferent animal species including man than any of the other species of the genus (Rich, 1951). *Mycobacteria* other than *Mycobacterium tuberculosis complex* are often referred to as "atypical" *Mycobacteria* or the Runyon's group. They are indeed, not species; rather each group comprises several species. There are four groups altogether and members are predominantly saprophytes with a few that can cause tuberculous like-disease (Pfeiffer, 2003). The groups which are lebelled I, II, III and IV and are named as follows:

Runyon group I. (Photochromogens). Members of this group include *M. kansasii, M. marinum*, and *M. simial*, which can cause tuberculous like-disease in man. They produce yellow to orange pigment when exposed to visible light.

Runyon group II. (Scotochromogens). Members of this group are slow growers; they produce yellow pigment when exposed to light or dark. The group is represented by *M. scrofulaceum, M. szulgai* and *M. gordonae*.

Runyon group III. (Non photochromogens) *M. avium complex* (4 serotypes), *M. intracellulare* and *M. xenopi* are species representative of this group. They have been associated with human infection. They do not produce pigment either in the dark or in the presence of light.

Runyon group IV. This group is made up of rapid growers, species include *M. fortuitum, M. chelonei* (Turtle tubercle bacilli) *M. smegmatis* and *M. phlei*.

Other specie in the group of Myccobacteria is *M. leprae,* the aetiologic agent of leprosy. It does not grow on any synthetic media and so can not be isolated in culture, but can be grown in armadillos (Collins *et al.*, 1993).

2.3 Characteristics of *Mycobacterium*

The organism is microaerophilic, acid fast, intracellular, non motile, non-spore forming, non-capsulated and gram-positive (Edwin *et al.*, 1974). *M. bovis*, commonly known as bovine tubercle bacillus, was first distinguished from other Mycobacteria through the work of Smith (1898) and Koch (1901). It is strongly acid fast bacillus whose rod (1-3 microns long) are sometime shorter and thicker than *M. tuberculosis*. Although *M. bovis* is Gram-positive, its cell wall composition is similar to Gram- negative organisms (Alhaji, 1976). The cell walls are unusual for their high lipid content (up to 60%) which is thought to account for their impermeability to stain, acid fastness, and resistance to disinfectants and for action by antibody –complement complex or bactericidal enzymes in phagocytic cells. The wax D of *M. bovis* differs from that of *M. tuberculosis* in lacking peptides, a fact which apparently makes it unsuitable for use as an adjuvant (Collins and Grange, 1983).

M. bovis grows more slowly on solid or liquid media especially on primary isolation than *M. tuberculosis*. *M. bovis* growth is inhibited by glycerol. An egg medium with pyruvate replacing glycerol is favourable for its growth, but the bacilli can grow either on egg or agar based medium (Alhaji, 1976). *M. bovis* can be differentiated from other Mycobacteria with a

simple biochemical test. They do not reduce nitrate to nitrite, niacin-negative, highly resistant to isoniazid (INH), and demonstrate a uniform resistance to pyrazinamide (PZA).

About 90% of all cases of tuberculosis were caused by *M. tuberculosis* complex, which is acquired (Michel 2002). Significant number of pulmonary tuberculosis can either be acquired from humans or cattle. Consumption of raw milk, close association between cattle and man, and inadequate or total lack of meat hygiene, account for exposure to infection by bTB (Alhaji and Schmurrenberger, 1977).

2.4 Epidemiology of Bovine TB Infection

M. bovis is one of the causative agents of tuberculosis that has a worldwide distribution and controlling the worldwide resurgence of TB requires understanding the route of transmission of the disease (Abubakar, 2007)

A Large number of domestic and wild animals and human populations have been known to harbour bTB, which contributes to the spread of the infection and also results in difficulty in its control (Pfeiffer, 1995). In some industrialised countries, *M. bovis* has been controlled in domestic animals, as a result of which the infection is rare (Michel, 2002). These countries are conscious of wildlife, local and international implications of the disease for trade in animals and animal products (Michel, 2002).

In Africa, where animals constantly live in the open, *M. bovis* had been rare but, introduction of European breed of animals and the subsequent development of intensive agriculture rapidly changed the distribution of the disease in these areas (Alhaji, 1976). The disease is now found in almost all African countries (Ayele *et al.*, 2004) affecting domestic animals and wildlife as well as humans. The disease was reported to be prevalent in 33 of 43 (80%)

African member countries of the Regional Commission of the Office International des Epizootics (World Organization for Animal Health, OIE)(Darbon and Grange, 1993).

Prevalence rate of *M. bovis* infection in cattle ranging from 2.5%-14% have also been reported from several studies in Nigeria (Alhaji, 1976; Eid, 1976; Shehu, 1988; Abubakar,1994; Du-Sai and Abdullahi, 1994; Nwchokor and Thomas, 2000; Cadmus *et al.*, 2004;2006;2007; Abubakar, 2007; Okaiyeto, *et al.*, 2008; Abubakar, 2010; Abubakar *et al.*, 2011; and Abubakar *et al.*, 2012).

2.5 Routes of Transmission for Bovine TB Infection

There are several routes of transmission for *M. bovis* in animals, but the primary routes of infection are via the respiratory and gastrointestinal tract (Thoen *et al.*, 2006). Experimental studies involving exposure of animals to *M. bovis* via different routes (intratrachial, oral, intravenous, intraperitoneal) have demonstrated that the nature and extent of tuberculous lesions vary with the route of exposure (Francis, 1971) and the location of tuberculous lesions affect how *M.bovis* is shed from the infected host. These include lesions of the respiratory system, kidneys, mammary gland and gastrointestinal system (Govier-Widen *et al.*, 2001). Externally draining cuteneous abscesses can also be a source of infection in a wide variety of domestic and wild mammals (Palmer *et al.*, 2003).

2.5.1 Inhalation

Respiratory transmission via the inhalation of contaminated aerosol or formites is the most efficient form of transmission, requiring a low number of organisms as an infective dose (Francis, 1971).Under most circumstances, an infected host generates an aerosol containing *M. bovis* when the animal coughs or sneezes, and the aerosol is inhaled directly by susceptible host, resulting in infection. Under natural conditions, respiratory transmission has been detected in herding animals such as domestic and wild animals as well as in captive herds of various cervid species (Schmitt *et al.*, 2002).

Transmission of *M. bovis* via inhalation is effective in animals that are kept in confinement and in free-ranging domestic and wild animals (Stetter *et al.,* 1995). Inhalation transmission has been demonstrated in different animal species. The lymph nodes associated with the respiratory tract, particulariy the bronchial and mediastinal, are the most commonly affected lymph nodes by the inhaled *M. bovis* (Palmer *et al.*, 2000).

2.5.2 Ingestion

Although respiratory transmission is the most important route of infection in animals that remain in close contact, oral transmission through ingestion of *Mycobacterium* is another important route. For transmission to be accomplished, susceptible animal has to consume feed or water contaminated with the mucous or nasal secretions, faeces or urine that contain the infective organisms or consume milk from an infected dam (Thoen *et al.*, 2006).

Oral transmission of *Mycobaterium* has been seen in several species through contaminated pastures (Serraino *et al.*, 1999), water source (Anonymous, 1994), infected animal carcasses

(Ragg *et al.,* 2000) and contaminated mucous or abscesses (Palmer *et al.,* 2000). Consumption of infected feeds has also been implicated in inter-species disease transmission

(Pfeiffer, 2003). In these circumstances, the mesenteric lymph nodes are usually affected (Govier-widen *et al.*, 2001) and have been used to suggest that oral transmission is more important route of infection in some situations (Palmer *et al.*, 2003).

2.5.3 Transcutaneous transmission

Another less common form of transmission is through transcutaneous means. This occurs in humans who handle infected carcasses where infection has been spread via cuts and abrasions (Grange and Yates, 1994). In animals, transcutaneous transmission is primarily from bites by infected animals. This has been seen in domestic cats (Ragg*et al.,* 2000), ferrets and European badgers (Gaveier-widen, 2001).

2.5.4 Vertical transmission

Vertical route of transmission is less evident in the epidemiology of *Mycobacterium* and rarely been documented in domestic animals in natural conditions (Philips *et al*., 2003). This form of transmission has been seen in many wildlife species, including badgers, brushtail possums and white-tail deer (Jackson, 2002)

2.6 Molecular Epidemiology of Bovine TB

Strain identification of the various species of *Mycobacterium* responsible for causing disease has been used for epidemiological investigation of tuberculosis and also for identification of potential sources of infection both in the general population and the nosocomial settings to help in developing better control measures. Recently, molecular techniques have replaced the phenotypic methods previously used and these are now a valuable tool in achieving this (van Soolingen *et al.*,1993; Durr *et al.,* 2000; Gori *et al.,* 2005). This has been in development since the mid-1980s leading to the availability of a number of genetic typing systems based upon the bacterial genome (genotyping) and has been actively explored. Considerable progress has so far been made in developing techniques that discriminate between isolates of *M. tuberculosis* complex (Durr *et al.,* 2000). Polymorphisms have been found to be present in a number of locations throughout mycobacterial genome, which are being studied and characterized in order to exploit them in epidemiological studies. For example, the insertion element IS6110 (Van Embden *et al.,* 1993), the polymorphic guanine cytocine-rich (GC-rich) repetitive sequences (PGRS) (Chaves *et al.,* 1996), the direct repeat region (DR) (Van Embden *et al.,* 1993; Van Embden *et al.,* 2000), major polymorphic tandem repeats (MPTR) and minisatelites, including the mycobacterial interspersed repetitive units (MIRUs) and the variable number of tandem repeats (VNTRs) (Frothingham and Meeker-O'Connell, 1988) are widely used for epidemiological studies.

However, detailed epidemiological investigation of bovine tuberculosis has been hampered by the lack of an established typing system for *M. bovis* (Durr *et al.,* 2000). This has been an impediment to understanding sophisticated epidemiological studies to assist in the control and eradication of tuberculosis in domestic animals. Though a lack of consensus on the best protocol still remains, progress is being made to develop standard protocols (Van Embden *et al.,* 1993). Researchers using the different techniques available for epidemiological studies of

mycobacterium have undertaken various studies. The advantages and limitations of various molecular techniques used in the study of the epidemiology of tuberculosis have been reported by many scholars.

2.7 Minimum Infectious Dose for *Mycobaterium* Infection

The minimum dose of *Mycobacterium* required for exposure in nature is unknown, but is likely to be highly variable and requires repeated exposure (Palmer *et al.,* 2006) and highly dependent on the route of infection. In numerous studies in guinea pigs as reviewed by Sigurdson (1945) infection through the oral route required doses orders of magnitude greater than that required for infection via aerosol. In fact, doses as low as 1–5 bacilli resulted in infection via aerosol, while 10–20 million bacilli were required to infect via the oral route (Palmer *et al.*, 2004a). There is great variation in the reported minimum infectious dose for cattle. Variations are due to differences in individual animals, host species, bacterial strain and route of inoculation. Studies reviewed by Francis (1947) showed that doses of 106–107 CFU were required to infect cattle or sheep through the oral route, while as few as 1–5 bacilli could infect via the aerosol route. Studies in cattle cited by Griffin and Dolan (1995) reported that 4 - 105 bacilli were required for infection via the respiratory route and 1000 times more bacilli were required for infection through the oral route. Recent investigations; however, have shown that animal can be infected orally with as few as 5000 CFU of *M. bovis* administered in the feed (Palmer *et al.*, 2004a). Conversely, mathematical modeling of *M. bovis* excretion by tuberculous animal suggests that a single bacillus may be sufficient to cause infection by the aerosol route (Neill *et al*., 1991).

2.8 Natural versus Experimental Infection

To simulate natural disease, experimental infection of cattle with *M. bovis* has been done by intranasal (Cassidy *et al.*, 1998, 1999), intratracheal (Buddle *et al.*, 1994) and intratonsilar routes (Palmer *et al.*, 1999b). Infections via the respiratory route in cattle likely mimics natural infection more than other methods of inoculation. However, there are various means by which inoculum may be introduced into the respiratory tract (e.g. intranasal, intratracheal aerosol). Intranasal administration of 104–105 CFU of *M. bovis* reliably results in lesions in the lungs and pulmonary lymph nodes. However, many other organs are also involved suggesting development of generalized tuberculosis (Neill *et al.*, 1988b). Moreover, lesions are also commonly noted in the nasopharyngeal mucosa and trachea; sites which are not commonly affected in naturally infected animals (Phillips *et al.*, 2003). Intranasal doses of 103–105 CFU have been suggested to be too high to simulate natural infection, resulting in atypical lesions in the upper respiratory tract and exaggerated involvement of the lungs (Phillips *et al.*, 2003). In one study, intranasal administration of 92 CFU of *M. bovis* did not result in lesion formation (Neill *et al.*, 1988b), whereas inoculation of 500–800 CFU resulted in lesions limited to the lungs and pulmonary lymph nodes (Buddle *et al.*, 1994, 1995). Inoculation using higher doses (e.g., 5-105 CFU) results in widespread lesions suggestive of generalized tuberculosis (Palmer *et al.*, 2004a).

Tracheal lesions, uncommon in natural infection, can also result as an artifact of the inoculation method. Intratonsilar inoculation of cattle with 1 - 105 CFU of *M. bovis* found to result in consistent early lesion formation in the medial retropharyngeal lymph nodes (Palmer *et al.*, 1999b) and later lesion development in the lung and pulmonary lymph nodes (Palmer, *et al.*, 1999a). Higher doses result in lesions of generalized tuberculosis. In aerosol exposure, the size of aerosolized droplets carrying *M. bovis* is as important as the dose of inoculum. The droplet nuclei mechanism of *M. tuberculosis* infection in humans has shown that residues, <5

mm, of evaporated droplets (droplet nuclei) can be generated by talking or coughing. Droplet nuclei remain airborne for prolonged periods while larger droplets settle within short distances of their source. Once inhaled, infectious droplet nuclei reach the terminal bronchioles and alveoli, while droplets >5 mm are removed in the upper respiratorypassages (Wells *et al*., 1948; Loudon and Roberts, 1967, 1968). Recent studies in cattle using aerosols containing *M. bovis* generated by a nebulizer have shown that nebulization of 1000 CFU of *M. bovis* can result in lesions similar to those seen in natural infection (Palmer *et al.*, 2002a). Lesions are limited to the lungs and pulmonary lymph nodes with no lesions in cranial lymph nodes, nasopharyngeal mucosa, or trachea. Moreover, *M. bovis* could only be isolated by bacteriological culture from the lungs and pulmonary lymph nodes when doses of 10^2 CFU were nebulized (Phillips *et al.*, 2003). Direct and indirect contact exposure have been successfully used to experimentally inoculate cattle and deer. In many cases transmission rates have been less than 100% with not all in-contact animals developing tuberculosis (Neill *et al.*, 1989; Palmer *et al*., 2004a), suggesting that the dose, although difficult to control, is not overwhelming. Moreover, the resulting distribution of lesions closely resembles that seen in nature in both cattle and deer. White-tailed deer whether exposed to *M. bovis* directly through shared housing (Palmer *et al*., 2001) or indirectly through shared feed (Palmer *et al*., 2004b), develop lesions similar to those seen in naturally infected deer (O'Brien *et al*., 2001). The lack of dosage control however, limits the utility of such a model in situations such as vaccine efficacy studies, where the assurance of a standardized dosage is critical.

2.9 Pathogenesis of Bovine TB Infection

Aerosol exposure of cattle to *M. bovis* is considered the most frequent route of infection; gross lesions usually involve the lungs and thoracic lymph nodes (Thoen and Bloom, 1995).

Cattle exposed by ingestion of food and water contaminated with *M. bovis* often develop primary foci in lymph tissues associated with the intestinal tract. Other Mycobacteria including *Mycobacterium* sub-specie *avium, Mycobacterium avium* subsp. *paratuberculosis, Mycobacterium intracelulare, Mycobacterium scrofulaèdum, Mycobacterium kansassi, Mycobacterium fortuitum,* and *M. tuberculosis* may induce tuberculin skin sensitivity, but they do not produce progressive pulmonary disease in cattle. Experimental investigations in animals comparing intravenous, intratracheal, intraperitoneal injection, as well as oral exposure to M. *bovis,* revealed that the nature and extent of disease varies with the route of exposure and dose of organisms (Vergne *et al.,* 2004). Tubercle bacilli were identified more than 100 years ago; however, definitive information on the pathogenesis of *M. bovis* in cattle and other bovidae is not available (Thoen and Barletta, 2004). Aerosol exposure leads to the involvement of the lungs and associated lymph nodes. The mucociliary clearance by mucus and epithelial cilia in the upper respiratory passages provides a defence against infection by inhalation of *Mycobacterium*. However, micro organisms on small particles such as dust and water droplets that do not impinge against the mucociliary layer can pass through terminal bronchioles, thus gaining access to alveolar spaces. Following aerosol exposure, *M. bovis* is carried to the small air passages, where it is ingested by phagocytes. The phagocytes pass through the lining of the bronchioles, enter the circulation, and are carried to lymph nodes, parenchyma of lungs, or other sites. After ingestion of the bacillus, the mononuclear macrophages attempt to kill the organism; however, virulent tubercle bacilli possess the ability to escape killing. Ingestion of the tubercle bacilli by the phagocytes into phagosomes or intracytoplasmic vacuoles protects the organisms from bactericidal components in serum. Following ingestion into phagocytes, mycobacteria effectively prevent phagolysosome fusion and acidification (Sturgill-Koszicki *et al.,* 1994).

Mycobacterial lipids such as lipoarabinomannan (LAM) and phosphatidylinosilolmannoside have been shown to intercalate within endosomal membranes and contribute to the arrest in phagosome maturation (Betty *et al.,* 2000; Vergne *et al.,* 2004). In addition, Mycobacterial proteins of the antigen *85* complex have been shown to localize within cytoplasmic vacuoles free of *Mycobacteria* (Betty *et al.,* 2000). By this mechanism, *Mycobacteria* survive and multiply within the phagosomes and eventually destroy the phagocytes. *Mycobacterium marinum*, a close relative of *M. tuberculosis and M. bovis*, may lyse the phagosome and enter into the cytoplasm and use actin polymerization to spread from cell to cell (Stamm *et al.,* 2003), a phenomenon that has not been observed with *M. tuberculosis* or *M. bovis*. Nonetheless, phagosomes containing *M. tuberculosis* or *M. bovis* (BCG strain) display certain degree of permeability, allowing entrance of cytosol components (Teitelbaum *et al.,* 1999). These findings led to the hypothesis that these membrane-permeable phagosomes may allow a bidirectional transfer of Mycobacterial products such as peptides, cord factor, or other toxic products from the phagosomes into the cytoplasm. This process may have implications for the role of cytotoxic T cells and class T mediated antigen presentation in the pathogenesis of Mycobacterial infections (Teitelbaum *et al.,* 1999). On the basis of these findings, pathogenic Mycobacteria may even gain access to the cytoplasm (McDonough *et al.,* 1993).

Following the stage described above, other phagocytes then enter the area and ingest the increasing numbers of tubercle bacilli. A small cluster of cells referred to as a granuloma develops. Cellular responses attempting to control the disease result in the accumulation of large numbers of phagocytes, and finally the formation of macroscopic lesions, denominated tubercles. After 10-44 days, cell-mediated immunity (CMI) responses develop and macrophages of the host have an increased capacity to kill the intracellular bacilli. The CMI responses are mediated by T lymphocytes, which release lymphokines (messenger proteins secreted by lymphocytes) that attract, immobilize, and activate additional

blood-borne mononuclear cells at the sites where virulent Mycobacteria or their products exist (Neill *et al.,* 1992). The cellular hypersensitivity that develops contributes to cell death and tissue destruction (caseous necrosis). In some instances, liquefaction and cavity formation occur as a result of enzymatic action on proteins and lipids. Rupture of these cavities into the bronchi allows aerosol spread of bacilli. Activated macrophages migrate to blind endings of lymphatic vessels and course to one or more of the thoracic lymph nodes, either bronchial or mediastinal (Lyashchenko *et al.,* 2004). Lymph nodes are more commonly infected than other tissues because fluids in an animal eventually pass through the nodes, where the meshwork of trabeculae entraps the Microorganisms. The enlargement and presence of macrophages in impenetrable passageways between reticular cell fibers of the lymph node provide an environment for Mycobacterial growth and development of the granulomatous lesion in the node (Neill *et al.,* 1994). On occasion, some phagocytized Mycobacteria remain in the lung, and both lung and thoracic nodes are affected. Primary lesions often become localized-in a node or nodes and may become large and firm (Neill *et al.,* 1989). Fibrous connective tissue development in the dynamics of granuloma formation probably contributes to the localization of lesions. Granuloma formation is an attempt by the host to localize the disease process and to allow inflammatory and immune mechanisms to destroy bacilli. A few lesions may appear to be regressing while becoming encapsulated by well organized connective tissue; however, such lesions may contain viable bacilli. Typically, the microscopic appearance of a granuloma (tubercle) is focal and has some caseous necrosis in a central area encircled by a zone of epithelioid cells, lymphocytes, and some granulocytes. Mineralization may be present in necrotic centers; in more advanced lesions, several foci of mineralization may coalesce. Multi- nucleated giant cells, which contain several nuclei, often in a horseshoe or ring shape near the cytoplasmic border, are often seen in the zone near the necrotic area. An outer boundary of fibrous connective tissue is usually present between the

lesions and normal tissue (Thoen *et al.,* 1988). Occasionally, fibrous tissue is not apparent, and the lesion assumes a more diffuse appearance. Lesions caused by *M. bovis* may have few, if any, organisms that can be found by microscopic examination ((Neill *et al.,* 1994).

2.10 Clinical Signs of Bovine TB Infection

The best detailed description of clinical signs of bovine tuberculosis is given in Hutyra and Marek (1913). Other descriptions have all been based on this original work. Francis (1947), Henning (1956) and Jubb and Kennedy (1970) have written revised and abridged versions of Hutyra and Marek's description. Radostits, (2010) gave a more general consideration to the disease, while Rich (1951) only gave passing consideration to the subject matter. Several articles have dealt with specific situations "based on case reports such as those by Kleeberg (1966) and McFarlane *et al.,* (1944). Due to the usually insidious nature of tuberculosis and the extraordinary variability of the lesions produced, the signs manifested frequently vary among individuals. In natural infection the incubation period is extremely variable, usually several months or years. Experimentally, the disease has been reproduced within two weeks using massive doses of the bacilli (Alhaji, 1976). Pulmonary tuberculosis, the most frequent form of the disease in cattle and humans, is characterized by progressive weight loss, frequent coughing, dyspnea, and sometimes diarrhea. Extension of infection to the pericardium leads to pericarditis and epicarditis.Tuberculosis of the abdominal organs of cattle, the second most frequent form, develops in an equally insidious manner (Alhaji, 1976). Radostits (2010) described reproductive disturbances in cattle as a result of extension of the disease to the reproductive organs. Cows may abort and bulls show epididymitis and testicular necrosis. Tuberculosis of the udder begins with a rather diffuse painless induration of one or both posterior quarters of the gland with gradual extension into the adjacent quarters. The

supramammary lymph nodes are invariably involved in tuberculosis of the udder, but they may sometimes be affected when the mammary gland appears normal. Milk production is usually normal, but as the disease progresses secretion gradually diminishes until it disappears entirely in the affected quarters. The milk is at first normal except at times, there may be flakes at the end of milking which are often indicative of tuberculosis (Radostits, 2010). Tuberculosis of the skeleton is usually hematogenous in origin and therefore initially an osteomyelitis occurs. It occurs most often in young cattle (Alhaji, 1976). The central nervous system involvement begins mainly as meningitis which may be a direct extension from vertebral osteomyelitis. Signs vary from loss of vision, incoordination or paralysis to just stiffness of quarters and the spine (Ayele *et al.,* 2004). In skin involvement, nodules of varying size appear singly or in groups, most commonly on the upper part of a limb. Subsequently they rupture and are transformed into fistulae or ulcers depending on the depth of the nodules (Ankugah *et al.,* 2002). Congenital tuberculosis in calves progresses quite rapidly and death usually occurs within few weeks or months. For congenital tuberculosis to occur, restricted infection must be present in the uterus (Jubb and Kennedy, 1970).

2.11 Lesion Distribution for Bovine TB Infection

With some exceptions, it is agreed that animals become infected with *M. bovis* by either the oral or respiratory routes and the oral route is likely most important in nursing tuberculous animals, while the respiratory route is most common in mammals in general (Neill *et al*., 1994).

In the late 1990's, surveys of tuberculous cattle in Great Britain (Phillips *et al*., 2003) revealed that 67% of tuberculous lesions were within the lungs and pulmonary lymph nodes

(tracheobronchial and mediastinal). Cranial lymph node lesions were observed in 39%, while only 8% of lesions involved mesenteric lymph nodes (McIlroy *et al.*, 1986).

Indeed, most studies of naturally infected animals describe the majority of lesions within the lung, pulmonary lymph nodes and cranial lymph nodes (Stamp, 1944; Lepper and Pearson, 1973). The conclusion is that this distribution of lesions suggests a respiratory route of infection. Investigations in naturally infected animals suggest that lesions of the alimentary tract, although not common generally, are more common in temperate climates where conditions favour dissemination and survival of *M. bovis* on forage, while respiratory tract lesions are more common in arid climates where conditions favour aerosolization of *M. bovis* (Lepper and Pearson, 1973).

In white-tailed deer and other cervids, lesion distribution differs from that seen in cattle. In white-tailed deer, elk (Cervuselaphusnelsoni), and red deer (*Cervuselaphus*) the preponderance of lesions occur in the medial retropharyngeal lymph nodes (Beatson, 1985; Whiting and Tessaro, 1994; Palmer *et al*., 2000); O'Brien *et al*., 2001). Similar to *M. bovis* infection of humans, but unlike tuberculosis in cattle, discharging sinus tracts have been associated with cranial lymph node lesions in red deer (Mackintosh and Griffin, 1994) and fallow deer (*Damadama*) (Robinson *et al*., 1989), but not white-tailed deer. Tonsilar lesions are commonly seen in white-tailed deer with tuberculous lesions of the medial retropharyngeal lymph nodes (Palmer *et al*., 2002b). In ruminants, afferent lymphatics are not present in the tonsil while efferent lymphatics drain from the tonsil directly to the retropharyngeal lymph nodes. This primary complex of tuberculous lesions involving the tonsil and retropharyngeal lymph nodes is remarkably similar to that seen in humans. In humans the primary complex resulting from oral exposure to *M. bovis* through consumption of unpasteurized milk consists of lesions in the tonsil and draining lymphnode (Ramsay, 1992; Samuelson and von Lichtenberg, 1994). This has led to the hypothesis that white-tailed

deer are generally infected with *M. bovis* through the oral route. Supporting evidence exists in demonstrations of deer to deer transmission through sharing of feed (Palmer *et al*., 2004b). In white-tailed deer, as well as other cervids, routes of inoculation that mimic oral exposure may be more relevant models of natural infection than those simulating aerosol exposure. Models of natural infection in red deer and white-tailed deer (Mackintosh *et al*., 1995; Palmer *et al*., 1999a) using intratonsilar inoculation result in lesion distribution similar to that seen in naturally infected deer (O'Brien *et al.,* 2001). Moreover, aerosol exposure of whitetailed deer to *M. bovis* did not result in lesion distribution similar to that seen in most naturally infected deer (Palmer *et al*., 2003).

It has been stated that the route of transmission of *M. bovis* can be deduced by the pattern of lesions observed in the infected animal (Biet *et al*., 2005). However, such a determination may not be straight forward.

A more recent study in cattle showed that feeding various doses of *M. bovis* resulted in lesions limited to the lung, pulmonary lymph nodes and cranial lymph nodes with no involvement of mesenteric lymph nodes or abdominal viscera (Palmer *et al*., 2004a). Furthermore, *M. bovis* could not be isolated from mesenteric lymph nodes or abdominal viscera by bacteriological culture. In this same study, interspecies transmission of *M. bovis* by sharing of feed between cattle and deer did not result in lesions of the mesenteric lymph nodes or abdominal viscera, but rather lesions of the lung, pulmonary lymph nodes and cranial lymph nodes. Critical to the assessment of route of infection through lesion distribution is the understanding of the primary complex. The primary complex of tuberculosis is considered to be the combination of lesions in the initial focus of infection and the regional (i.e. draining) lymph node (Palmer *et al*., 2003). In cases of prolonged or severe disseminated disease, determination of the primary complex may be impossible; confusing speculation concerning the initial route of infection. Moreover, when infection occurs across

mucous membranes such as the pharynx or intestine, the initial lesion in the mucous membrane may not be visible while lymph node lesions are obvious (Dungworth, 1993).

2.12 Shedding of *Mycobacterium*

Accurate quantitative assessments of shedding of *M. bovis* from infected cattle are difficult. In humans, repeated sputum samples are evaluated before a diagnosis is reached. In cattle, most investigations have involved single time-point samples. In some cases, *M. bovis* infection has been confirmed by detailed postmortem examination, including bacteriologic culture (McIlroy *et al.*, 1986), in others, however, no such examination was conducted (De Kantor and Roswurm, 1978). Estimates from such studies suggest that 9–19% of infected cattle shed *M. bovis* in nasal or tracheal secretions. Experimental studies to examine shedding document latent periods between inoculation and initiation of shedding. This shows that latent period appears to be inversely related to inoculum size. Cattle receiving 104 CFU of *M. bovis* intranasally shed *M. bovis* in nasal mucus in 17.6 days, while those receiving 106 CFU intranasally shed *M. bovis* in nasal mucus in 11 days (Neill *et al.*, 1988a). Once initiated, shedding is intermittent for up to 38 weeks.

Many investigators have categorized TB in cattle as either open or closed (Lyashchnko *et al.*, 2004). Open cases of tuberculosis are those in which lesions, primarily in the lung; allow excretion of *M. bovis,* while closed cases are categorized by lesions that do not promote excretion of bacilli. It is artificial to categorize cases as open or closed, as many cases do not present with obvious gross pulmonary lesions, yet contain small lesions that are only detectable after meticulous examination, including microscopic examination. Careful examination revealed that 73% of bovine cases with pulmonary lymph node lesions also had lesions in the lungs (McIlroy *et al.*, 1986). In 63% of the cases only one lung lesion was

present and 70% of the lung lesions were <1 cm in diameter. The authors conclude that all tuberculous cattle with lesions in the pulmonary lymph nodes should be considered capable of excretion of *M. bovis.* In a separate study of tuberculin positive cattle, although all had gross lesions of the pulmonary lymph nodes, only one animal had gross lung lesions. *M. bovis* was found by bacteriological culture; however, in 4 of the 25 animals, demonstrating that gross lung lesions were not a prerequisite of bacillary shedding (Neill *et al*., 1988a). Moreover, animals with no visible lesions are not necessarily non-infected. *M. bovis* has been isolated from skin-test negative animals with no gross lesions (Neill *et al*., 1992). Meanwhile, as many as 10% of cattle with no visible lesions can be culture positive for *M. bovis* (Corner, 1994).

2.13 Bovine TB Infection in Domestic Animals other than Cattle

There is some degree of specific host susceptibility to the various species of *Mycobacterium*, although cross infection are known to occur, with animal species being more susceptible to *M. bovis* while man on the other hand is more susceptible to *M. tuberculosis* (Alhaji, 1976; Pfeiffer, 2003). Susceptibility to *M. bovis* by other species of animals other than cattle has been reported by several authors. For instance, it has been demonstrated that small ruminants (Lall, 1969; Cousins, 1998) and pigs (Snider, 1971; Pfeiffer, 2003) are susceptible to *M. bovis.* Similarly cats and dogs (Radostits *et al.*, 2010) as well as horses have been shown to be susceptible (Francis, 1958; Morris *et al.*, 1994).

2.13.1 Bovine tuberculosis in camels

M. bovis infection in camels was reported by several authors (Littlewood, 1989; Lingard, 1905; Leese, 1908; Mason, 1917; Wernery *et al*., 2007; Abubakar, 2010). In 1917, Mason reviewed earlier reports on tuberculosis in camels and recorded his own observations. It appeared that tuberculosis in camels was uncommon. Of 7411 aged camels slaughtered in the Cairo abattoir from 1910-1916 inclusive, 218 (2.9%) were tuberculous (Mason, 1917). He stated that all tubercle bacilli isolated by him from dromedary camels were of the bovine type (Mason, 1917). He also stated that in Egypt, camels were often kept in close association with cattle and the route of infection was usually the respiratory tract. In 274, he had seen, the lungs and bronchial lymph nodes were mostly affected and in 60% of cases, lesions were restricted to these organs. Generalized tuberculosis, with very numerous lesions in the principal organs and carcase lymph nodes, was observed in about 7% of cases. Tuberculosis due to *M. bovis* was reported in a breeding herd of 19 Bactrian camels *(Camelus bactrianus)* (Bush *et al.*, 1990). Also tuberculosis infection was diagnosed in 13 alpacas on two different farms in the Netherlands in 1988 (Anon, 1989). The presumed low prevalence report of *M. bovis* infection in camelids is combined with the low specificity of the diagnostic test result and a very poor predictive value for a positive test and reluctance by camelid owners to cull animals which test positive (Bleem *et al.,* 1993).

2.13.2 Bovine tuberculosis in free- ranging wildlife and captive animals

Infection with *M. bovis* has been documented in wildlife throughout the world and in most situations these cases have been considered to be spill over from infected domestic populations (LoBue *et al.,* 2003). Cases have been reported in Europe (Clifton-Hadley *et al.*,

1995), Africa (Michel, 2002), Asia (Kanameda and Ekgatat, 1995), Australia (Mclnerney et al., 1995), Newzealand (Montgomery, 1999) and USA (Nishi, *et al*., 2002).

In recent years, several free-ranging wildlife reservoir hosts have been identified. These species include North American bison (Nishi *et al*., 2002), African buffalos (Michel, 2002), European badgers (Clifton-Hadley *et al.*, 1995), Newzealand brushtail passums (Jackson, 2002), white-tail deer in Mechigan (Schmitt *et al.,* 2002) and several antelopes in South Africa (Michel, 2002). Studies on these wildlife reservoir hosts for *M. bovis* are providing greater insight on the epidemiology of *M. bovis* in both wild and domestic animals population, which is needed to allow more effective control of the infection in domestic livestock.

The occurrence of *M. bovis* in captive wildlife populations has been well documented and the epidemiology of the disease in captive populations is very similar to circumstances seen in domestic animals. A wide variety of species have been documented to be infected with *M. bovis*, including non-human primates and elephants (Keet *et al*., 2000). Surveillance and TB control programmes for captive wildlife are similar to those exercised for domestic animals.

2.14 Bovine Tuberculosis in Humans

Human infection with *M. bovis* is a recognized public health hazard in developing and industrial nations (Grange and Yates, 1994). The advent of milk pasteurization and eradication programs has reduced the level of human *M. bovis* in industrialized nations, but sporadic cases still occur when individuals come in contact with infected livestock (Pfeiffer, 2003), captive wildlife (Stetter *et al.*, 1995) or contaminated animal carcasses (Pfeiffer, 2003). Humans are susceptible to *M. bovis,* and there are numerous instances of human

infection resulting from contact with infected animals. Recently there has been increased interest among public health officials, in drug-resistant strains of *M. tuberculosis, M. bovis* and *M. avium*, because several of such strains have been isolated from HIV/AIDS infected and immunocompromized humans (Pfeiffer, 2003). Infection with *M. bovis* causes pulmonary and extrapulmonary disease (Grange and Yates, 1994). Contact with infected animals is a source of *M. bovis* infection for humans and is a recognized hazard for abattoir workers, veterinarians and livestock handlers (Stetter *et al.*, 1995).

2.15 Diagnostic Tests for Bovine Tuberculosis Infection

The intradermal tuberculin test using purified protein derivative *(PPD)* prepared from the culture filtrate of *Mycobacterium bovis* by precipitation with ammonium sulfate or trichloro acetic acid is widely used to identify tuberculous cattle and other animals in countries throughout the world (Haagsma and Angus, 1995; O'Reilly, 1995; Kaneene and Thoen, 2004).

The protein content of the PPD is determined and biologic activity evaluated in sensitized guinea pigs. The single caudal fold intradermal test, the single cervical intradermal test, and the comparative cervical test are the tuberculin tests used in most bovine tuberculosis control or eradication programs (O'Reilly, 1995; Kaneene and Thoen, 2004). In the United States, the caudal fold test is a presumptive test. The injection site is visually observed and palpated at 72 hours following injection of *M. bovis* PPD, and any inflammatory response is classified as suspect. Cattle with suspicious reactions to the caudal fold test are subsequently subjected to a comparative cervical test to determine their relative responsiveness to

biologically balanced *M. bovis* and *M. avium* PPD (Roswurm and Konyha, 1973; Duffield *et al.,* 1985).

Cattle infected with *M. bovis* will develop more induration (measured as an increase in skin thickness at the site of injection) in response to the *M. bovis* PPD than to the *M. avium* PPD, except in animals with advanced disease that have become non responsive to tuberculin. The increase in skin thickness at each injection site is plotted on a scattergram. The results are classified as negative, suspect, or reactor (positive) (Roswurm and Konyha, 1973).

In vitro cell-mediated assays that monitor cell responses to PPD by incorporation of tritiated thymidine or the production of gamma interferon have been developed and evaluated in cattle and other animals in several countries (Muscoplat *et al.,* 1975; Wood *et al.,* 1992). In addition, *in vitro* assays have been developed to detect antibodies in *M. bovis* infected cattle (Thoen *et al.,* 1975; Thoen *et al.,* 1980). The tuberculin skin test, which involves the injection of 0.1 ml PPD intradermally and observation of the injection site at 72 hours in cattle, requires the handling of cattle twice. An *in vitro* blood test would provide a distinct advantage because animals would be restrained only once; this is especially important when working with wild animals (Thoen *et al.,* 1980; Thoen *et al.,* 1992). The specificity and sensitivity of a diagnostic test should be determined in naturally sensitized *M. bovis* infected cattle and in cattle in *M. bovis free* herds in the geographical region under conditions in which the test will be used (O'Reilly, 1995; Kaneene and Thoen, 2004; Adams, 2001).This is important because non-specific sensitization caused by other organisms that share antigenic determinants may vary for different regions and countries (Karlson, 1962). For example, it is possible that cattle not infected with *M.*

bovis will respond to *M. bovis* because of exposure to other Mycobacteria (i.e., *M.avium subsp paratuberculosis)*

2.15.1 *In vitro* tests for bovine TB infection

The primary immune response in animals to *M. bovis* infections is cell mediated (Thoen and Barletta, 2004). Because some concerns have been reported about the sensitivity and specificity of the tuberculin skin test, considerable research has been conducted to develop *in vitro* correlates of delayed hypersensitivity to monitor cell-mediated responses (Muscoplat *et al.,* 1975; Wood *et al.,* 1992). Initial efforts involved the development of lymphocyte blastogenic assays (LBA) using the incorporation of tritiated thymidine following exposure to specific mycobacterial antigen as a measure of cell-mediated responsiveness (Muscoplat et al., 1975). The stimulation index is determined by comparing the response to specific antigen to non stimulated controls and is used to determine the status of the animal. Investigations conducted on experimentally infected cattle revealed a good correlation with LBA and skin tests (Thoen, *et al.*, 1980). However, the results of LBA and skin test responses in cattle naturally exposed to *M. bovis* varied considerably in different herds within the same geographical region. Systematic studies to determine the conditions of shipment and storage time failed to provide definitive information useful in explaining the variation in findings on LBA. The reproducibility of LBA on the same animals was questioned because results sometimes varied even when conditions for shipment and storage were similar. Therefore, the LBA has not come into widespread use as a stand-alone or supplemental diagnostic test to be used routinely in the diagnosis of bovine tuberculosis (Wood *et al.*, 1991).
More recently (Muscoplat *et al.*, 1975; Wood *et al.*, 1991), a gamma interferon (IFN) assay was described for monitoring cell-mediated responses. This *in vitro* assay is similar to the LBA in that it requires the collection of blood samples from cattle for antigen stimulation. It differs from the LBA in

that IFN produced by the stimulated cells is measured in an ELISA, and no radioactive isotopes are involved in measuring cell stimulation. The IFN assay has been used in detecting tuberculous cattle in several countries, and the results correlate with responses obtained on a tuberculin skin test (Wood *et al.,* 1992; Whipple *et al.,* 1995; Ryan *et al.,* 2000). This test has been approved as a supplemental test for cattle in the U. S. National Tuberculosis Eradication Program. Nevertheless, a comparison of the sensitivity of the caudal fold test and the IFN assay revealed that the caudal fold test was significantly more sensitive than the IFN for the diagnosis of bovine tuberculosis (Whipple *et al.,* 1995). Maximum overall sensitivity was achieved when results of the caudal fold test and IFN assay were interpreted in parallel. The specificity of the IFN *assay* has been reported to be 93%; however, the probability that a positive test indicates infection (i.e., the positive predictive value) is reduced when testing cattle in low-prevalence herds. This positive predictive value can only be improved by increasing the specificity of the IFN assay. As with most *in vivo* assays, the success for detecting *M. bovis* infected animals and minimizing false-positive responses varies with different antigen preparations. *M. bovis* PPD and *M. avium sub avium* PPD are used to stimulate lymphocytes in the plasma sample to produce IFN. The use of proteins secreted by *Mycohacteria* such as ESAT-6 may improve the specificity of the IFN assay (Pollock and Andersen, 1997; Waters *et al.*, 2004). Numerous investigations have been conducted to develop antibody-based diagnostic tests (e.g., ELISA) for detecting *M. bovis* infected cattle and other animals (Thoen *et al.*, 1975; Thoen, 1988; Ritacco *et al.*, 1990; Sugden *et al.*, 1997). These tests could be used in conjunction with tuberculin skin tests or. *in vitro* cell-mediated tests to identify tuberculous animals with advanced disease that fail to elicit a response on intradermal testing with PPD (Plackett, 1989). The sensitivity and specificity of serologic tests may be improved by using purified antigens (Pollock and Andersen, 1997). However, it is generally agreed that antibody-based tests are not stand-alone tests, as they fail to detect many *M.* bovis infected cattle with subclinical disease in a population (Kaneene and Thoen, 2004; Thoen *et al*., 1975). Moreover, nonspecific responses may be observed on these tests because of responses elicited by other mycobacteria or organisms that share antigens with *M*

bovis. Development of more sensitive and specific serologic tests through continued research could be useful in slaughter surveillance programs and when evaluating progress within individual herds. However, unless sensitivity and specificity are increased dramatically, they would only be considered presumptive tests, and the intraderinal and *in vitro* cellular assays would be used in making a final diagnosis.

The use of the intradermal tests, along with herd management, has been successful in eradicating tuberculosis within herds. However, to allay concerns about the specificity of the tuberculin skin tests, *in vitro* cellular assays may improve diagnostic specificity when used in conjunction with the intradermal tests. The *in vitro* tests have the advantage that animals only need to be handled once and the animals can he retested at short time intervals. However, the cost of IFN assay may limit its use. SD-biolineTB Ag MPT64 is a rapid immunochromatographic identification test for the *Mycobacterium tuberculosis* complex that uses mouse monoclonal anti-MPT64. This test kits can be easily used for rapid identification of the *Mycobacterium tuberculosis* complex in combination with culture system based on liquid or solid media without any technical complexity in clinical laboratories as described by the manufacturers. The test cassette consists of a sample pad, a gold conjugate pad, a nitrocellulose membrane and an absorbent pad. Mouse monoclonal anti-MPT64 was immobilized on the nitrocellulose membrane as the capture material (test line). Other antibodies, which recognized another epitope of MPT64, conjugated with colloidal gold particles were used for antigen capture and detection in a sandwich type assay.

SD Bioline TB Ag MPT64 Rapid test device has a letter of T and C “Test line and Control line” on the surface of the case. Both the “Test line and the Control line” in result window are not visible before applying any samples. The “Control line” is used for procedural control. Control line should always appear if the test procedure is performed properly and the test reagents of control line are working. As the test sample in the sample well flow laterally through the membrane, the antibody-colloidal gold conjugate binds to the MPT64 antigen in the sample. The complex then flows further and bind

to the mouse monoclonal anti-MPT64 on the solid phase in the test line, producing red to purple colour band. In the absence of MPT64, there is no line in the test band region.

It is important to emphasize that to confirm a diagnosis of *M. bovis* infection, it is necessary to isolate and identify the acid-fast organism by biochemical tests or molecular techniques (Kaneene and Thoen, 2004).

2.15.2 Restriction fragment length polymorphism (IS6110-RFLP)

The most important insertion sequence identified in the *M. tuberculosis* complex is the IS6110. IS6110-RFLP is the most widely used and standardised molecular method for differentiating between *M. tuberculosis* isolates (Van Embden *et al,.* 1993). The IS6110 insertion sites in the chromosome and the copy number varies for different strains of *M. tuberculosis* from zero up to 20 copies present in the genome (Plate 2.1) (Brosch *et al.,* 2000a). For instance, there are 16 copies present in the genome of *M. tuberculosis* H37Rv and only four copies in the CDC1551 strain. This resulting degree of polymorphism has enabled IS6110-RFLP to be used as a genetic marker for epidemiological studies, thus making it the 'gold standard' for molecular epidemiological studies of human tuberculosis (Van Embden *et al.* 1993; Gordon *et al.,* 1999b). Generally, strains from outbreaks or patients with common transmission route will display identical banding patterns.

However, fewer copies of IS6110 are present in *M. bovis* than M. tuberculosis with the majority of bovine isolates having only a single copy, which is located at the direct repeat (DR) region (Collins *et al.,* 1993; van Soolingen *et al.,* 1994; Cousins, 1998). Nonetheless, the use of IS6110-RFLP is recommended to fingerprint *M. bovis* isolates for epidemiological purposes, where the population of the *M. bovis* contains multiple copies of IS6110 (i.e. more than 3 copies) due to the good discrimination offered by the technique. However, if a majority of the isolates contain 3 or fewer copies of IS6110, additional sub-typing methods are required (Durr, 2000).

The transmission of TB can be investigated and the results compared between different laboratories both locally and internationally through the use of IS6110-RFLP. Van Soolingen and colleagues(1995) discovered the prevalence of a single *M. tuberculosis* genotype in various East Asian countries known as the “Beijing family” when they used IS6110-RFLP to investigate the population structure of *M. tuberculosis* strains in China and Mongolia.

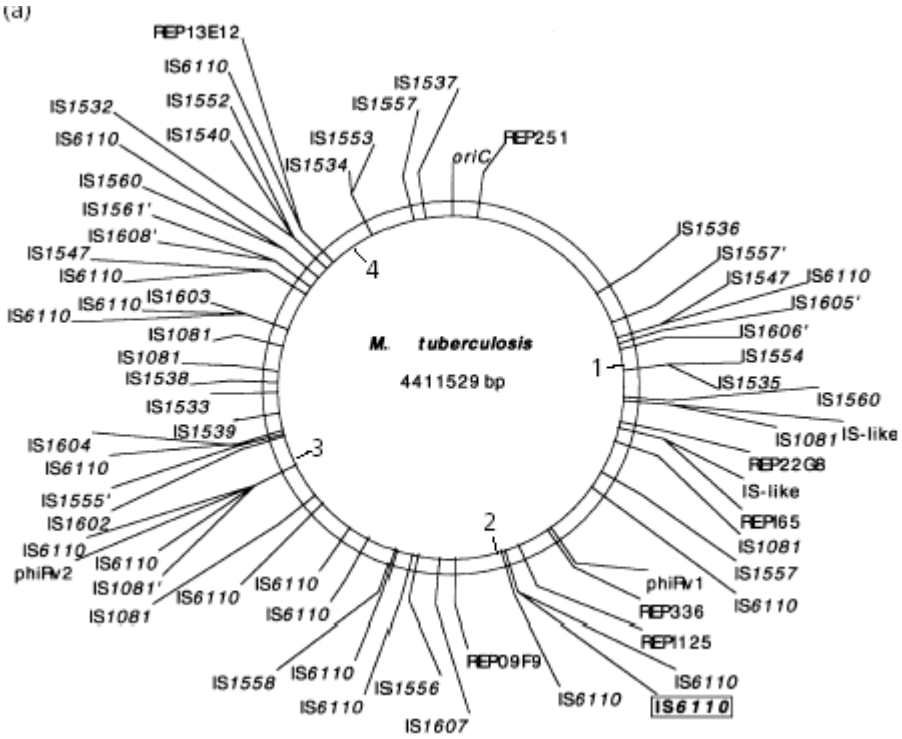


Plate: I: Distribution of the IS6110 insertion sequence on the *M. tuberculosis* chromosome (Brosch *et al.,* 2000a)

However, the method has some disadvantages, which include the need for large quantity of good quality DNA; it is a technically demanding and expensive method. The method also requires a longer period to get results; it is less discriminatory for isolates with low copy numbers and less reliable (Cave *et al.,* 1994). In such cases a second genotyping technique such as spoligotyping is often used to establish polymorphisms between strains of isolates with low copy number (van Soolingen, 2001). It is also possible for isolates to have identical DNA fingerprints without being epidemiologically

linked, as the IS6110 copies are not distributed randomly throughout the genome, but insert themselves at hotspots (McHugh and Gillespie, 1998) (Plate 2.I)

2.15.3 Restriction endonuclease analysis

This method was the first to be developed for intra-specie typing of *M. bovis* (Collins and Delisle, 1985). As the only typing technique available in the late 1980s and early 1990s, REA was used for molecular epidemiological studies in New Zealand and Ireland (Collins and de Lisle, 1986). However, Collins *et al.*, (1993) reported that the method is technically demanding and interpretation of the complex REA pattern is difficult (Collins *et al.,* 1993; Durr, 2000). But, it plays a good role in interspecies typing and also has great potential for classifying closely related species (Collins *et al.,* 1985). The method has no means for numerically cataloguing types, which makes comparison between laboratories difficult. However, in New Zealand, REA still remains the technique of choice due to familiarity and superior discrimination as compared with new techniques (Collins, 1999).

2.15.4 Pulsed field gel electrophoresis (PFGE)

The problem of excessive number of small DNA fragments encountered with REA was solved by the development of PFGE (Durr, 2000). The steps are basically the same with only difference in the use of endonulease enzymes to generate small number of large DNA fragments. Zhang *et al.,* (1995) published one of the earliest reports on the use of this method for the typing of *M. tuberculosis,* and later for differentiation of *M. bovis* Bacillus Calmette-Guerin (BCG) substrains. Feizabadi *et al.,* (1996) also reported a good description between types for isolates of *M. bovis*, unfortunately the application of the technique on *M. tuberculosis* complex is difficult and labour intensive.

2.15.5 Polymorphic guanine and cytosine-rich repetitive sequences (PGCRS)

Mycobacterium has very high guanine-cytosine content and within the genome, short repeated sequences are present which have a G-C composition in excess of 80% (Poulet and Cole, 1995a; Cole *et al.,* 1998). These polymorphic sequences are present in multiple clusters scattered throughout the genome. The use of PGRS-RFLP has been demonstrated to have a good degree of strain differentiation of *M. bovis* (Cousins *et al.,* 1993). It is recommended that PGRS-RFLP is the method of choice for maximum sensitivity in subdividing strains of *M. bovis* that have a single or low copy numbers of IS6110 (Cousins and al e 1998). The PGRS elements appear to be quite stable in *M. tuberculosis*, because an identical PGRS-RFLP fingerprint was reported to be identical over a 3.5 year period from a single patient (Chaves *et al.,* 1996). However, this technique is found to be time consuming and technically demanding and cumbersome; consequently, variable results can be obtained even by the most experienced technician (Durr, 2000). The method also requires large amount of DNA and hence the need for culturing samples which can cause delay and inconvenience when investigating the epidemiology of an outbreak when results are required quickly.

2.15.6 Spoligotyping

This method is known as 'spacer oligonucleotide typing' and represents the first PCR based technique to be widely accepted. It is based on DNA polymorphism present at one particular chromosomal locus, the "Direct Repeat" (DR) region, which is uniquely present in *Mycobacterium tuberculosis* complex bacteria (Plate 2.2). It is found between the 36bp direct repeats (DR) in the genomic DR region of the *M. tuberculosis* complex strains (Goyal*et al.,* 1997). This locus was first described by Hermans *et al.,* (1991) who sequenced this region in *Mycobacterium bovis* BCG, the strain used worldwide to vaccinate against tuberculosis. The DR region in *M. bovis* BCG consists of directly repeated sequences of 36 base pairs, which are interspersed by non-repeatitive DNA

spacers, each 35 to 41 base pairs in length. The spacer sequences of the DR region are amplified by PCR using labelled primers that anneal to the end of the DRs and then hybridised to a membrane that has oligonucleotides, which correspond to various spacer sequences bound to it. By spoligotyping one can detect the presence or absence of spacers of known sequence. The 43 spacer sequences used are derived from 37 *M. tuberculosis* H37Rv and six *M.* bovis BCG P3 strains. Individual *M. tuberculosis* complex isolates contain various numbers of these sequences and these can be used to differentiate them by the patterns of positive and negative hybridisation signals (Goyal *et al,.* 1997). The function of these DR loci is however unknown.

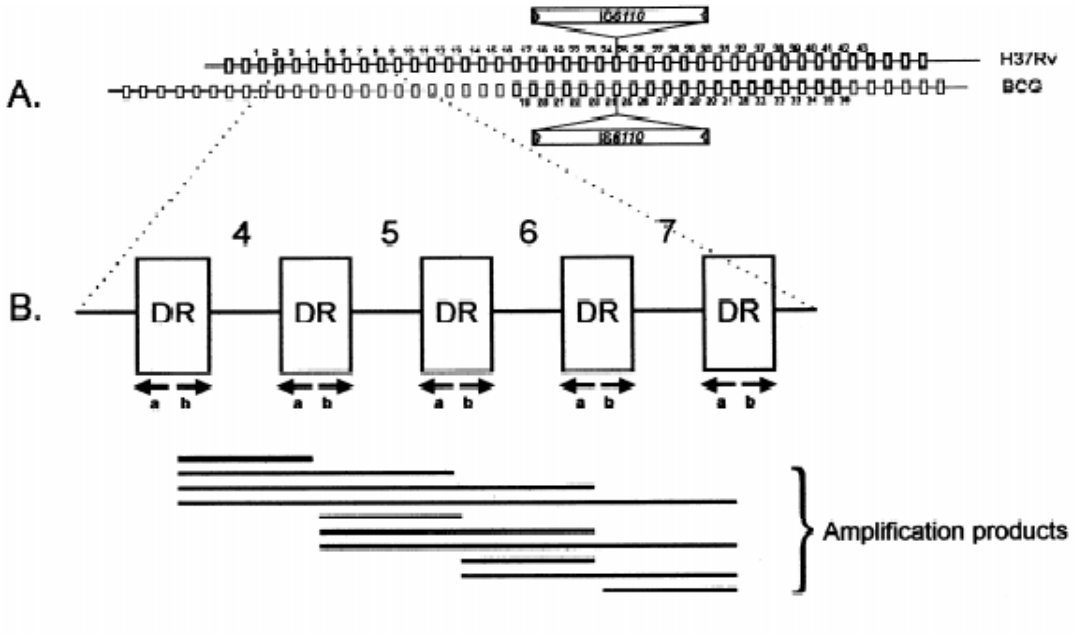


Plate: II: Structure of Direct Repeat loci in the *Mycobacterium* genome (Kamerbeek *et al.,* 1997)

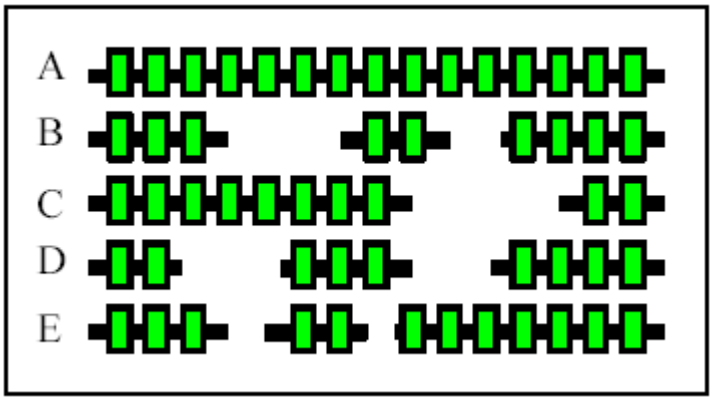


PLATE: III: Schematic presentation of the polymorphism in DR regions of different *M. tuberculosis* complex strains. (Kamerbeek *et al.*,1997)**.**

The chromosomes of *M. tuberculosis and M. bovis* contain 48 and 41 DRs respectively which are interspaced with unique spacers varying in length from 35 to 41 bp(Kamerbeek *et al.* 1997). The polymorphism seen between clinical isolates at this locus is mainly due to the presence or absence of one or more direct repeats plus the adjacent spacer, known as the direct variant repeat (DVR) (Plate III)

Spoligotyping is an additional typing method for those members of *M. tuberculosis* complex isolates with fewer copy numbers of IS6110 (Goyal *et al.,* 1997). The value of this method for strain differentiation has been assessed in several studies (Durr, 2000). Aranaz *et al.,* (1996) have shown that the technique did enable the grouping of the affected host specie, which presumably reflected an underlying difference in epidemiology of the disease but differentiation of isolates was poor compared with IS6110-RFLP. Similarly, it has been demonstrated that spoligotyping is particularly useful in eliminating the suggestion of transmission between patients who are epidemiologically linked, but was also found to be less discriminatory than RFLP (Goyal *et al.* 1997; Goyal *et al.,* 1999). Bauerl (1999) used spoligotyping as an additional method to type *M. tuberculosis* strains with low IS6110 copy numbers and found that spoligotyping has higher discriminatory power than IS6110 typing. For their study, 249 isolates were typed using both methods (spoligotyping and IS6110-RFLP) and IS6110-RFLP produced 56 patterns, 16% of which were unique while the remaining 83% were clustered into 16 groups. Eighty-seven different spoligotype patterns were observed with 25% of all isolates having unique spoligotypes whilst the remaining 75% were clustered into 24 groups. This study showed that spoligotyping is very useful as a secondary typing method especially for isolates with low IS6110 copy numbers. Recently, Gori *et al.,* (2005) also compared spoligotyping and IS6110-RFLP and demonstrated spoligotyping sensitivity of 97.6% with specificity of 47%.

Spoligotyping, allows getting a first good picture of strain identity in a new and more localized or confined setting and its useful in case of epidemic spread. It is recommended as a valuable first step in analysing single or low copy number of *M. bovis* strains (Cousins *et al.,* 1998). The method is particularly useful to simultaneously detect and type *M. tuberculosis* complex bacteria directly from clinical samples; therefore there is no delay since the time wasted for culture is not there (Goyal *et al.* 1999). It is also an easy and fast method compared to IS6110 RFLP and the patterns are easy to read and compare via computer software.

However, some spoligotype patterns may be indistinguishable and difficult to interpret, which could be due to chains of recent transmission causing isolates to cluster, or it could also represent broad

genetic similarities between isolates which share a more distant common ancestor (Hayward and Wartson, 1998). The main disadvantage of spoligotyping is that all genetic polymorphism is restricted to a single genomic locus, which is the DR cluster. It has also been reported that the method can result in overestimation of recently transmitted disease (Gori *et al.,* 2005).

2.15.7 Mycobacterial interspersed repetitive units (MIRUs)

MIRUs are DNA elements ranging from 40-100bp in size dispersed throughout the intergenic regions of the *M. tuberculosis* complex genomes. MIRUs are thought to be involved with regulation of gene expression, differential translation of genes within a polycistrotic operon and some may function as structural components for chromosome organisation (Supply *et al., 1*997). Supply *et al.,* (2000) have identified 41 of such loci in the *Mycobacterium tuberculosis* H37Rv genome and have termed them mycobacterial interspersed repetitive units. They are also shown to contain 65 copies of these repeats whining these 41 loci; twelve of which display differences in tandem repeat copy numbers and sometimes in the sequences of the repeat units (Brosch *et al.* 2000a), which make them similar to human mini satelite variable number tandem repeat (VNTR) regions.

MIRU-VNTR PCR based method is based on the variable number tandem repeats of MIRUs. The isolates are typed by the number of copies of repeat units found at the loci; which is determined by the size of the fragment produced by amplifying the entire locus using primers that anneal to the flanking DNA (Mazars *et al.,* 2001), and the repeat units are known to be between 52 to 77 base pairs (bp) in length.

MIRU-VNTR typing produced more distinct patterns when compared with IS6110 RFLP and spoligptyping (Barlow *et al.,* 2002). In a study of 180 *M. tuberculosis* and *M. bovis* isolates with low IS6110 copy number between six and zero, Cowan *et al.,* (2002), found that MIRU-VNTR method produced 80 distinct MIRU patterns with 6 isolates possessing unique MIRU patterns and 120 grouped into 20 clusters; while IS6110-RFLP typing produced 58 patterns in total. Thirty-eight of the

isolates had unique patterns, whilst 142 were grouped into 20 clusters. On the other application of spoligotyping on the same samples produced 59 distinct spoligotype patterns with 43 unique patterns and 137 isolates grouped into 16 clusters. Combining the three methods led to maximum specificity in typing. MIRU-VNTR has the advantage of rapid turn around hence can be automated for large-scale typing projects and it does not requires large quantity of DNA (Supply *et al.,* 2000; Sola *et al.,* 2003)

2.15.8 Variable number tandem repeat (VNTR)

Genetic loci containing variable numbers of tandem repeats (VNTR loci) form the basis for human genetic mapping. The technique is also used for human forensic and paternity testing (Goyal *et al.,* 1994; Frothingham, 1995). VNTRs are hypervariable loci within the genome of tandemly repeated DNA sequences, which vary in copy number at a given site between different individuals of a population resulting in allelic variation. This variability is thought to be generated by slippage during replication, which results in the newly synthesized strand having extra or missing repeat units in comparison with the original template (van Belkum *et al.,* 1998).

The technique is also PCR based and primers are designed to anneal to the DNA flanking the VNTR locus, so that after amplification the polymorphism in the number of repeats can be examined by gel electrophoresis. Conservation of flanking DNA is generally good between strains, and can sometimes be observed among different species (van Belkum *et al.,* 1998). This allows the analysis of the polymorphisms at the VNTR loci to be investigated across genus.

Many possible VNTR loci have been studied in isolates of *M. tuberculosis* and *M. bovis* (Frothingham *et al.,* 1988; Goyal *et al.* 1994; Skuce *et al.,* 2002). Almost 42 VNTRs have been identified that could be used for differentiation of *M. tuberculosis* complex strains. These include the exact tandem repeat (ETRs) (Goyal *et al.,* 1994), the major polymorphic tandem repeat (MPTR) (Supply *et al.* 2000) and also the sets of Queen's University Belfast (QUB) VNTRs (Roring *et al.,* 2002). ETR-A, ETR-B, ETR-

C, ETR-D, ETR-E are the most common ETR for strain typing with ETR-A being the most discriminatory compared with the others (Sola *et al.,* 2003).

In a study conducted by Skuce *et al.,* (2002), on 100 *M. bovis* isolates, the technique identified 33 allele profiles for the six novel VNTRs in comparison to 29 profiles produced by spoligotyping on the same isolates. VNTR typing was also reported to be more discriminatory than IS6110-RFLP in another study conducted by Spurgiesz *et al.,*(2003). In another study of 461 isolates of *M. bovis* from Northern Ireland, Skuce and colleagues (2005) reported that VNTR assay discriminated 40 different profiles, with the most prevalent constituting 21 per cent of the total while spoligotyping discriminated only 14 profiles.

VNTR typing has the advantage of being highly discriminatory and reproducible among laboratories, and the data can be displayed in a simple numerical format that is easy to manage (Skuce *et al.,* 2005). However, deletions and insertions within the repeat are also observed at some VNTR loci, this causes ambiguities in the definition of the size coding between studies (Le Fleche *et al.,* 2002).

Though there are various techniques for genotyping, the choice for an epidemiological study depends on the particular circumstances such as study of global, national or local transmission patterns. It also depends on cross contamination, or investigation, of local outbreaks. Each of these require a different combination of techniques (Durr *et al.*, 2000; Kaduma *et al.*, 2003). However, cost and availability of infrastructure may be important in selecting techniques in developing countries like Nigeria. Nonetheless, a choice of one or more relatively cheap and less technically demanding techniques might be incorporated to achieve maximum strains discrimination in these areas.

2.15.9 Genotype MTBC

The majority of mycobacterial infections are caused by strains of the *Mycobactrium tuberculosis* complex (MTBC). MTBC includes the species *M. tuberculosis*, *M. africanum*,

M. bovis, M. microti M. canettii and M. pennipedii (Cousins *et al.,* 2003). The *M. bovis* species can be further divided in to *M. bovis subsp. bovis, M. bovis subsp. caprae* and the *M. bovis*-derived BCG vaccine strain, whereas *M. africanum* includes two subtypes, 1 and ll (Pfeiffer *et al.,* 2003). According to (Elvira *et al.*, 2004; Romero *et al.*, 2007 and Neonakis *et al.,* 2007) identification of mycobacterial to the species level on the basis of growth rate, phenotypic characteristics and biochemical test is laborious and extremely time-consuming. Geno-Type MTBC (HainLifescience, Nehren, Germany) is a recently developed DNA-srtip assay for differentiating MTBC strains isolated from clinical and cultured material. The procedure involves isolating DNA from clinical or cultured material, multiplex amplification with biotinylated primers and revars hybridization of the single stranded, biotin-lebeled amplicons to membrain-bound probes. The resulting banding pattern indicates the species of the isolated mycobacterium. The assay permits genetic differentiation of the following species: *M. africanum 1, M. bovis BCG, M. bovis subsp. bovis, M. bovis subsp. caprae, M. microti, M. tuberculosis, M. africanum* (Elvira *et al*., 2004). Although the species are closely related genetically, they differ in host and geographic range, certain phenotypes and pathogenicity. *M. tuberculosis* is the most significant pathogen for humans in Europe and America, whereas *M. africanum* is widely distributed among African patients (David *et al.,* 1978). Both subspecies of *M. bovis* are reported to infect humans, yet they have a broad host range, including wildlife and domestic livestock (Kubica *et al.*, 2003). Of particular interest intrinsic resistance of *M. bovis subsp. bovis* against pyrazinamide, one of the first-line antituberculous drugs (Konno *et al.,* 1967). The vaccine strain *M. bovis* BCG is more frequently used for bladder cancer immunotherapy and can be detected in human urine specimens from bladder cancer patients (Brandau and Bohle, 2001). Thus, for those specimens, the isolation of *M. tuberculosis* complex is not necessarily an indication for antituberculous treatment. Therefore, rapid differentiation to the species and subspacies levels

should be obtained not only for epidemiological purposes but also for adequate treatment for each patient (Elvira *et al.,* 2004).

2.16 Control and Prevention of Bovine Tuberculosis

According to Cousins *et al.,* (2001) there are various reasons for attempting to eradicate bovine TB which includes; the risk of infection to the human population, loss in productivity due to infected animals and animal market restrictions set by countries with advanced eradication programmes. The priority they are giving will vary depending on factors specific to the country in question. In Africa, the economic losses associated with livestock contracting bovine TB has either never been studied or has not been examined sufficiently (Cousin *et al.,*. 2001). However, data can be obtained from other countries where bovine TB is prevalent. In Argentina, for example, the annual loss due to bovine TB is approximately US$63 million (Cosivi, *et al.,* 1998). Based on these estimates, it can be inferred that bovine TB may pose a serious economic risk to Africa. Moreover, preliminary reports submitted by member states to the OIE indicate that the disease occurs almost everywhere on the African continent, with inevitable economic and public health implications (Cosivi, *et al.,* 1998). In industrialised countries, control and eradication of bovine TB has been successfully carried out by regular testing and removal of infected animals under mandatory national bovine TB programmes. Such programmes have been successful in many European Union member states and in seven central European countries between 1953 and 1980 (Pavlik *et al.,* 2002). In developing countries, however, bovine TB remains a major animal health problem, mainly

because these countries cannot shoulder the financial burden required to implement a control programme and compensate for slaughtered animals. Limited access to education, poor information networks and lack of disease surveillance are other factors that limit the implementation of any such programme.

2.16.1 Vaccination

BCG, an attenuated strain produced by continuous subculture of a wild-type *M. bovis* isolate from cattle, has played a crucial role in controlling human TB, particularly in children. However, its use for bovine TB is less effective. Using BCG vaccination to control bovine TB is an option that has been considered in European countries, North America and some African countries. However, because of its limited effectiveness, the Joint WHO/FAO Expert Committee on Zoonoses stated in its second report that 'The committee was of the opinion that vaccination has no place in the eradication of bovine TB in cattle (WHO, 1959). Attempt to protect cattle against bovine TB by BCG vaccination had no success (Waddington and Ellwood, 1972). Buddle *et al.,* (1995) found an absence of protective immunity in BCG-vaccinated cattle, possibly linked to immune responses developed to environmental Mycobacteria, although it might have been expected that exposure to the shared antigens of environmental Mycobacteria would provide acquired protection from bovine TB. A study conducted by Corner *et al.,* (2002) on the use of vaccine against bovine TB in brushtail possums (*T. vulpecula*) revealed 69% efficacy of BCG vaccine. Skinner *et al.,* (2003) examined the efficacy of vaccination with BCG alone and a DNA prime-BCG boost regimen in cattle challenged with virulent *M. bovis.* The prime-boost regimen significantly enhanced protection in six parameters compared to significant enhancement of protection in only two

parameters for BCG alone. This was demonstrated by fewer animals with severe lung lesions, fewer lymph nodes with lesions per animal, a smaller proportion of animals with lesions, lower mean lung and lymph node lesion scores and less *M. bovis* isolated from retropharyngeal and thoracic lymph nodes compared to non-vaccinated challenge animals (Corner *et al.*, 2002). A vaccination strategy employing BCG would obviously necessitate developing differential diagnostic assays to distinguish vaccinates from non-vaccinates, as BCG vaccination causes sensitivity to tuberculin, the PPD routinely used in skin testing tuberculous cattle. Because of this and the varying efficacy of BCG in cattle, recent research capitalising on advances in immunology and molecular biology has focused on alternatives to BCG, including novel attenuated *M. bovis* strains, sub-unit vaccines and recombinant DNA vaccines (Ginsberg, 2002). The recently available *M. bovis* genome sequence should have a significant impact on new generation vaccine candidates (Garnier, *et al.*, 2003). Eradication of bovine TB using compulsory test and slaughter strategies has proven difficult even in industrialised countries, where cattle movement can usually be controlled. In some of these countries, where natural reservoirs of the disease in wild animals pose a serious risk of transmission to domestic livestock, the problems have been exacerbated, and eradication programmes remain unsuccessful. In most African countries, controlling free movement of animals within a country is difficult and movement between countries cannot be regulated, primarily due to a lack of border controls. In addition, spread of bovine TB amongst wildlife in game parks in Africa is increasingly being recognised as a serious problem, with consequences for domestic animals (Daborn and Grange, 1993). Therefore strategic vaccination of susceptible domestic animals in endemic areas is a feasible option for Africa, where control of bovine TB is a much more acceptable and practical measure rather than eradication of the disease as suggested by (Daborn and Grange, 1993). Skinner *et al.*, (2003) reported that vaccination could potentially be used to control bovine TB in countries where

wildlife reservoirs exist and in those that cannot afford conventional control procedures. Development and production of an effective vaccine with appropriate methods and strategies for delivery could therefore contribute to bovine TB control in Africa. This is obviously a task best undertaken at a global level and applied locally in appropriate scenarios.

2.17 Bovine TB in Nigeria

The prevalence of bTB infection based on studies conducted in Nigeria has indicated a steady presence over the last 39 years (1976 and 2015). For instance, Alhaji (1976) reported a 2.5% prevalence rate in northern region of Nigeria while in the same year Eid (1976) reported a 1.5% prevalence rate for cattle in the north western part of Nigeria. Similarly, Ayanwale (1984) reported 7.8% infection rate in a study in some Southern states of Nigeria, Shehu (1988) reported 11.8% infection rate in Kaduna state while Abubakar (2007) reported 14% prevalence rate in Kaduna and FCT. Other such studies both in Nigeria and other African countries have shown a similar trend over the years (Ellwood 1975; Abubakar 1994; Du-Sai *et al.* 1994; Jiwa *et al.* 1997; Vekemans *et al.* 1999; Shirima *et al.,*2003; Cadmus *et al.,* 2004, Abubakar 2010). Added to the high costs of sustainable control programme are problems of social unrest due to political instability and ethnic wars especially among the local farmers and the Fulani herdsmen. It could also be as result of intercontinental cattle movement due to lack of proper border control as a result of insufficient collaboration with bordering countries and the smuggling of live animals and hence lack of quarantine (Abubakar, 2007).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Safety

All fieldwork carried out in this study had safety undertaken and necessary precautions taken to avoid injury to the researcher, assistants and the animals.

3.2 Study Area

3.2.1 Borno State

Borno State is located in the arid zone in the North Eastern corner of Nigeria. This is within latitude of 10-13^{0}N and longitude 12-15^{0}E. It has boundaries with Chad to the North East, Cameroon to the East and Adamawa to the South West. The area falls in the tropical continental north with a dry season of 4-8 months of duration (October to May) followed by a short (4 months) rainy season (late June to early October) (Gambo *et al.*, 2010). According to the 2006 census, the population of the State estimated to be 4,558,668 (Gambo *et al.*, 2010).

3.2.2 Yobe State

Yobe State located in North Eastern part of Nigeria. It was carved out of present-day Borno State, with an area of about 45,502 km^2 and lies within latitude 11-12^{0}N and longitude 10-13^{0}E within the Savannah region of Nigeria. According to the 2006 census, the population of the state estimated to be 2,532,395. The state borders the Nigerian States of Bauchi, Borno, Gombe, and Jigawa. It borders the Diffa Region and the Zindar Region to the north in The Republic of Niger. Because the state lies mainly in the dry belt, the state is dry and hot for most of the year, except in the southern part of the state which has a milder climate. The area falls in the tropical continental north with dry month of between 4-8 months (October to May) followed by a short (4 month) rainy season (late June to early October) (Yobe State Dairy, 2007).


YOBE
BORNO
JIGAWA
KANO
KADUNA
BAUCHI
NIGER
PLATEAU
FCT
OYO
KOGI
BENUE
TARABA
LAGOS
EKITI
DELTA
EBONYI
ENUGU
CROSS RIVER
BAYELSA
RIVERS
AKWA IBOM
ANAMBRA

Figure: 3.I. Map of Nigeria Showing Borno and Yobe States

Anounymous: http://www.informationnigeria.org/2013/09/9:31AM

3.3 Study Design

A cross-sectional study was performed beween 2011 and 2012 in Borno and Yobe States, within the savanna region of Nigeria. All fieldworks were carried out in the five zonal veterinary divisions (testing areas) of each of the two states for tuberculin testing and post-mortem meat inspection while the laboratory work (staining, culture, isolation and identification) were carried out at the tuberculosis and HIV laboratory of Zankli Medical Center, Abuja, Nigeria.

3.3.1 Sampling procedures

The cattle herds tested were derived from the two states, in the Sahel part of Northern Nigeria. In each state, five zonal veterinary testing areas were selected based on judgemental or purposive sampling method. This was done in consultation with the Directors of Veterinary services of the two states. Only cattle above one year of age were tested. Cattle were grouped into four age groups (>1 - 2yrs, >2 - 4yrs, >4 - 6yrs and >6yrs). (Conservative age of individual animal was determined based on history from the herdsmen and/or Dental examination) (Abubakar, 2007). Animals selected for the study were grouped into two, based on management systems, the semi-settled herds (migratory pastoralist) and the settled herds (non migratory and often is near a large town). This is because they are the main sources of

meat, milk and milk products to the public. Free treatment for minor illnesses, de-wormers, acaricides and CBPP vaccination were provided by the states as incentives after taking the final reading of tuberculin test result (Appendix 1).

3.3.2 Cattle restraint

Pastoralist cattle herds were always in the open field with no animal handling facilities. All cattle were paired before testing started. One pair of cattle was handled at a time. Three to four people were required per pair, one man applied head restraint to the animal not being tested, while the rest of the men restrained the animal being tested using a rope on the head and a milker's hobble on the rear legs. All restraint procedures were performed by the pastoralist themselves because the cattle were more familiar with them and the pastoralist preferred to do it themselves. In Government or private herds where a crush was available, it was used for restraint.

3.3.3 Cattle identification

The majority of cattle tested had no permanent identification. Oil based paint was used to serially number the cattle in each herd. The paint was applied on the back after the animals were restrained. The numbers were large enough that they were easily read for at least one week after application.

3.3.4 Tuberculin test procedure

Tuberculin testing was conducted using purified protein derivative (PPD) obtained from Prionics Lelystad, Netherlands to screen cattle for *M. bovis.* The technique used was the single intradermal caudal fold test (SCF) applied as described by Lesslie (1975); Alhaji, (1976); Monaghan (1994). A 2ml automatic syringe with 26G needle was used. A distance of

5-7cm away from the base of the tail on the left site was cleaned with dry cotton and 0.1ml of bovine PPD tuberculin was carefully deposited intra-dermally.

3.3.5 Reading and interpretation of the test

The test was read 72 hours post- injection, the restraint procedures for the reading was as described for the application of the test. The technique of reading the test has been described by Alhaji, (1976). The injection site of each animal was examined both visually and by palpation. All responses to the tuberculin test were recorded on tuberculin test form. D-symbols were used for difuse response while N-symbols were used for circumscribed or nodular responses. For this study, in which most of the herds tested had no previous tuberculin test recorded, all reactions, nodular or difuse were interpreted as positive. All animals with no reaction to the tuberculin test were interpreted as negative.

3.4 Collection and Handling of Bovine Tissue Samples

A total of four thousand one hundred and thirty (4130) cattle were examined for bovine TB-like lesions between 2011 and 2012. Tissue samples were taken from slaughtered animals with lesions, which were suspected to have or be compatible with bTB. The samples were taken from lymph nodes (retropharyngeal, bronchial and mediasternal), lung, intestines, kidneys, liver or any other organ with lesions such as the heart, spleen and mammary gland. Epidemiological data such as age, sex, body condition scores and locations of lesions were taken from each carcass sampled. The samples were obtained from five major abattoirs that supply meat to the public in each of the two states (Borno and Yobe). These organs were examined visually for changes in colour, (pale yellow or gray) or morphology and then palpated before incision from the surrounding tissues. Samples were obtained in sterile

screw-capped containers and transported, on ice, to the laboratory where they were frozen until processed (Appendix 2).

3.5 Questionnaires

A total of 500 structured and close ended questionnaires were distributed or administered to Cattle handlers such as cattle rearers, cattle marketers, butchers and meat inspectors to assess knowledge, awareness, attitude and practices of the groups regarding bTB (Appendix 4). Respondent were selected based on judgemental or purposive sampling method.

3.6 Collection and Handling of Human Sputum Samples

3.6.1 Ethical consideration

Approval was obtained from the Ethics Committees of all participating hospitals and TB centres in the Hospital Management Boards of the two States prior to the study (Appendix 5).

3.6.2 Demographic details of the TB patients

Suspected TB patients from the two States were enrolled with their informed consent and the following details were recorded: name, age, sex, occupation, history of BCG vaccine in the past, history of contact with animals and consumption of locally produced milk 'nono' with or without HIV infection (Appendix 3).

3.6.3 Human sputum collection

A total of Three Hundred and Ninety One (391) Sputum samples were collected from clinical suspected TB patients that attended five human hospitals in each of the two States involved, WHO's Directly Observed Therapy, short-course strategy (DOTS) centres, as well as designated TB centres within the study area. Three sputum samples were collected from each patient i.e. 1st spot, overnight and 2nd spot as recommended by the International Union Against Tuberculosis and Lung Diseases (IUATLD) (Ipuge, Riedaer and Enarson 1996). Samples were collected in sterile screw capped containers with CetylPyridnium Chloride (CPC) as a preservatives and decontaminants, transported to the laboratory in ice parks for processing.

3.6.4 Proforma forms

All data obtained from TB patients prior to sample collection were in a form of proforma forms (appendix 3). These forms were analysed to identify percentages associated with sex of TB patients, history of prolong close contact with cattle and consumption of locally produced milk ''nono'' and with or without HIV

3.7 Laboratory Procedures

3.7.1 Preparation of Lowenstein and Jensen media (LJ)

The technique used was as described by (Joshua *et al.,* 2013)

a. Mineral Salt Solution:

Potassium dihydrogen phosphate	-	2.4g
Magnesium sulphate	-	0.24g
Magnesium citrate	-	0.6g
Asparagine	-	3.6g
Glycerol/Pyruvate	-	4.5g
Distilled water	-	600mls

b. Egg Fluid:

Eggs were cleansed in 5% soap solution and rinsed well in running water, placed in 95% ethyl alcohol for 5 minutes, removed and broken into a sterile beaker container, yolk and white were mixed well by shaking, then filtered through sterile gauze and mixed well.

c. Malachite Green Solution

1 percent malachite green was desolve in distilled water

Malachite green - 1gm

Distilled water - 100mls

i) To each 1600mls of prepared substances (1 litre egg fluid + 600mls mineral salt solution) 40mls of malachite green was added.

ii) This was then distribute in 10mls amounts into sterile plastic universal screw-capped bottles

iii) Then slanted and inspissated at 85^{o}C for 50 minutes.

iv) Followed by incubate at 37^{o}C for 48 hours to check sterility and stored at -4 to -6^{o}C with caps tightly closed.

3.8 Laboratory Processing of Bovine Tissue Samples (Culture)

Tissue samples were decontaminated prior to culture as described in the Veterinary Laboratory Manual (Anonymous 1970; Corner 1988, Abubakar, 2007).

1. The sample was removed from the freezer and submerged in diluted locally produced household bleach (Jik, 3.5% NaOCl) (Reckitt Benkiser, Nigeria Ltd). It was left at room temperature before rinsing with freshly diluted bleach for culture.
2. Specimens were thawed at room temperature and then trimmed of excess tissue and fat.
3. Tissue samples were homogenized (individually) in a sterile blender for 2mins in 50ml nutrient broth containing phenol red.

4. Five milliliters of the aliquot homogenate was transferred into 50ml screw capped centrifuge tube and 20ml of 4% NaOH was added and shaken for few seconds.
5. The sample was allowed to stand for 40mins at room temperature before adding 6NHCL drop-wise to neutralize until first colour change (purple to pink or muddy-pink) and then centrifuged for 30mins.
6. The supernatant was discarded leaving about 2mls, which was mixed with the pellets and inoculated on two Lowenstein-Jensen slants, glycerol and pyruvate enriched, using sterile pipette applicator.
7. The tubes were then incubated at 37°C for a minimum of 8 weeks (Anonymous 1970; Parra 2005; Abubakar, 2007).

3.8.1 Primary isolation

Cultures were examined weekly for colonies with a hand lens; the growth time and colonial characteristic were noted. A representative colony was smeared and stained by the Zeil Nelsen stain technique for presence of acid-fast bacilli (AFB) and cellular morphology was noted.

3.8.2 Acid-fast /Zeihl-Neelsen (ZN) staining

Zeihl-Neelsen staining was carried out using standard protocol as described by Joshua *et al.*, (2013) to detect acid-fast bacilli.

1. An impression smear was made using new, clean and labelled grease-free slide and the slide was air-dried and heat fixed by passing it through a flame (Over a bursen-bunner) with the specimen side up. This is to fix the specimen to the slide and preserve the bacterial morphology.
2. The slide was then flooded with carbolfuschin and then steamed gently with the flame from underside. It was then rinsed off with water and decolorized with 5% acid alcohol until the red colour is gone.

3. The slide was rinsed again with water and counter-stained with methylene blue.
4. Additional rinsing with water was applied to remove excess colour and air dried. It was then examined under a microscope with oil emersion lens at x 100 to look for acid fast bacilli (AFB). The bacilli appear red, straight or slightly curved rods occurring either singly or in groups while non-acid-fast microorganisms stained blue.

3.9 Laboratory Processing of Milk Samples (Culture)

Milk obtained from individual cows that were tested tuberculin positive, purchased for post-mortem meat examination. Primary mycobacterial isolation was undertaken using Lowenstein-Jensen media (Becton-Dickenson, UK) and culture procedures were carried out according to standard methods as described in the manual of clinical laboratory procedures (Anonymous 1970).

1. Forty milliliters (40ml) of milk sample was placed in a 50ml centrifuge tube and centrifuged at 3000g for 15mins.
2. The supernatant was discarded and the residue re-suspended with 20ml 4% NaOH containing phenol red indicator.
3. The suspension was allowed to stand for 30mins before neutralizing by drop-wise addition of 6NHCL until the colour changed from purple to pink.
4. It was then centrifuged again at 3000g for 20mins and the supernatant was discarded.
5. The sediment was inoculated on two Lowenstein-Jensen media (glycerol and pyruvate enriched slants).
6. The slants were incubated at 37°c for a minimum of 8 weeks.

3.9.1 Primary isolation was done as earlier described

3.9.2 Acid-Fast/Zeihl Nelsen staining ZeihlNeelsen staining was done as earlier described

3.10 Laboratory Processing of Human Sputum (Culture)

1. Sputum samples were preserved and decontaminated using the CetylPyridnium Chloride (CPC) before inoculating on to LJ-slopes using standard method (Nwfor, 2011; Joshua *et al.,* 2013). To an Xml of sputum (depending on the quantity available), 15ml sterile saline was added and the mixture was then allowed to stand for 15mins at room temperature with occasional shaking before centrifuging at 3000g for 15mins.
2. The supernatant was poured off and 20ml sterile saline was added to re-suspend the sediment before centrifuging again for another 15mins.
3. The supernatant was decanted and the sediments inoculated onto 2 slants of Lowenstein-Jensen media (glycerol and pyruvate enriched) before incubating at 37°c for a minimum of 8 weeks.

3.10.1 Primary isolation was done as earlier described

3.10.2 Acid-Fast/Zeihl Nelsen staining ZeihlNeelsen staining was done as earlier described

3.11 Handling and Processing of Mycobacterium Isolates

3.11.1 SD-biolineTB Ag MPT64

3.11.2 Test procedure

1. 3 – 4 colonies were suspended in 200ul of extraction of buffer prior to test
2. The test device was removed from the foil pouch and placed on a flat, dry surface
3. 100ul of suspended solid cultures in buffer were added in to the sample well
4. As the test begins to work a purple colour moved across the result window in the centre of the test device
5. Result was interpreted in 15 minute after sample application

3.11.3 Interpretation of the test result

1. A colour band appears in the left section of the result window indicating that the test is working properly. This band is the control line (C)
2. The right section of the result window indicates the test result (T)
3. The presence of only one purple band within the result window indicates a negative result.
4. The presence of two purple bands ('T' band and 'C' band) within the result window indicates a positive result.
5. If the purple band colour is not visible within 15 minutes after performing the test, the result is considered invalid. It is recommended that the specimen be re-tested.

*****Marketed as (SD-TB Bioline Ag MPT64)

3.12 Genotype® MTBC Assay for Molecular Identification of MTBC Species

The GenoType MTBC assay is based on MTBC specific 23S ribosomal DNA fragment, *gyr*B DNA sequence polymorphisms and RD1 deletion. The assay was performed according to the manufacturer's instructions and as previously described (Richter *et al.,* 2003; Romero *et al.,* 2007) that involved three technical steps.

3.12.1 DNA extraction by chemical method

a. 300µl of distilled water was pipetted in 1.5mls screw capped tube, scrap a few loops (2 or 3) of the MTB culture on Lowenstein Jensen (LJ) slant and the colonies were emulsified into the distilled water in screw cap tubes.
b. Briefly vortexed to mix and 100µl lysis buffer was added.
c. Incubated for 5-8 minutes at 95^0C in a water bath.
d. 100µl of neutralizing buffer was added and vortexed for 5 second.

e. Spined down for 5 minutes at full speed.

f. 5-10µl of the supernatant was directly used for amplification (PCR).

3.12.2 Amplification reaction

a. The mixture was prepared by adding 35 µl of PNM (containing a mixture of triphosphate deoxynucleoside and primers marked with biotin),

b. 5µl of PCR buffer,

c. 2µl of 2.5 mM $MgC1_2$,

d. 0.2 µl of Taq DNA polymerase

e. 3µl of distilled water was added

f. 5µl of DNA preparation was added to this mixture to reach a final volume of 50µl.

g. Instruments, racks and bench space were decontaminated with freshly prepared 0.5% hypochlorite solution before and after loading the thermo-cycler.

h. The reaction tubes were placed or loaded on to thermo-cycler.

i. On the thermo-cycler, appropriate program was selected for the amplification procedure.

j. Amplification was performed using the following amplification protocol: Denaturation cycle of 15 min at 95^0C, followed by by 10 denaturation cycles 30 S at 95^0C and 10 elongation cycles for 2 min at 58^0C, followed by 20 additional denaturations of 25 S at 95^0C and annealing of 40 S at 53^0C, continuing with an elongation step of 40 S at 70^0C, and finishing with an extension cycle of 8 min at 70^0C.

k. At the end of this amplification procedure, the amplicons obtained were used for hybridization and detection.

3.12.3 Hybridization

In the hybridization steps, probes are embedded in the strips which will compliment the correct DNA sequence, if present in the amplicons. The primers used in the amplification process are biotinylated. When amplicons are subjected to all conditions as prescribed in the hybridization procedure, complimentary sequences will be visible as bands on the strips. These bands are further interpreted to define positive diagnosis or absence of susceptible TB, MTBC (*Mycobacterium tuberculosis* Complex).

The following protocol describes the manual hybridization using TwinCubator;

a. New gloves and lab coat worn, working area deconterminated with freshly prepared 5% sodium hypochlorite.
b. Hybridization buffer (HYB) and Stringent wash (STR) were pre-warmed at 45^0C to dissolve all crystals.
c. Rinse solution and distilled water were brought to room temperature
d. CON-C and SUB-C were freshly diluted; 1:100 in the respective diluents provided in the kits and protected from light.
e. 20µl of denaturation solution (DEN, blue) was dispensed in a corner of each of the well used.
f. 20µl of amplified sample solution was added, pipetted up and down to mix well and incubated at room temperature for 5 minutes.
g. 1ml of pre-warm Hybridization Buffer (HYB, green) was carefully added to each well and the tray was gently shaken until the solution has a homogenous color.
h. Strip was placed on each well.
i. The tray was placed on TwinCubator and incubated for 30 minutes at 45^0C.
j. Hybridization Buffer was completely aspirated using pipette.

k. 1ml of Astringent wash solution (STR, red) was added to each strip and incubated for 15 minutes at 45^0C using TwinCubator.

l. Astringent wash solution was completely removed

m. 1ml of Rinse solution (RIN) was added to each strip and incubated for 1 minute

n. RIN solution was completely removed

o. 1ml of diluted conjugate (For dilution 10µl CON-C + 990µl CON-D) was added to each strip and was incubated for 30 minutes

p. Conjugate was completely removed.

q. Each strip was washed with 1ml of Rinse Solution twice for 1 minute and was washed once with 1 ml of distilled water.

r. 1 ml of diluted Substrate (For dilution 10µl SUB-C + 990µl SUB-D) was added to each strip and incubated for 2 – 10 minutes and protected from light without shaking.

s. Substrate was completely removed

t. Reaction was stopped as soon as bands were clearly visible by rinsing twice with distilled water for 1 minute in each rinse.

u. The DNA strips were removed from the tray and air dried between two layers of absorbent paper

3.12.4 Evaluation and interpretation of result

Strips were pasted and protected from light; an evaluation sheet was used for the interpretation of result. Developed strips were pasted in the designated fields by aligning the bands CC and UC with the respective lines on the sheets. Species were determined with the help of the interpretation chart and inter name of the identified species. Each strip has a total of 13 reaction zones (Plate 3.1).

1. The first band contains the conjugate control designed to indicate that the conjugate has been effectively united with the substrate, thereby facilitating correct visualization.
2. The second band includes a universal control designed to detect all known mycobacteria and members of the group of gram-positive bacteria with a high G+C content. This band is used for checking the presence of the amplified product after hybridization.
3. The third band contains a sequence that amplifies a fragment of the 23S rRNA region, which is common to all known members of the *M. tuberculosis* complex.
4. Amplification band 4 – 13 includes probes specific for each of the tuberculosis complex species.
5. The combination of these bands enables identification of the different species within the complex.

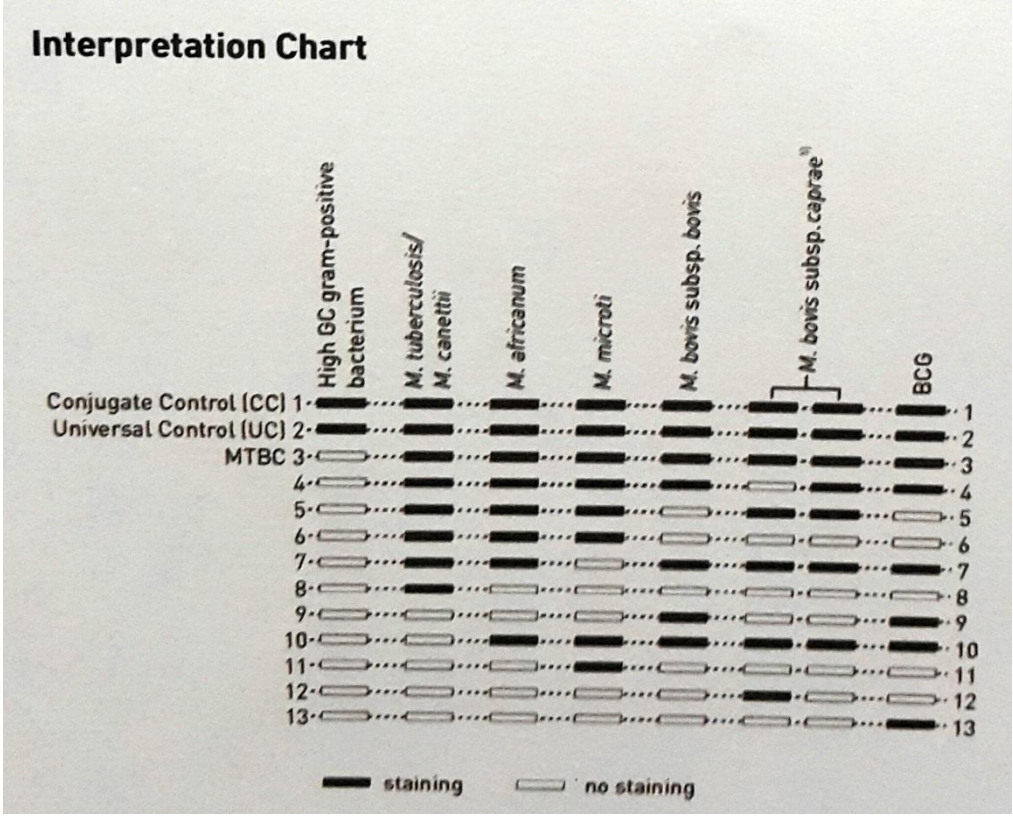


Plate IV: Interpretation chart for identification of MTBC species

3.13 Data Analysis

The chi-square (χ^2) was used to calculate the expected values with their appropriate degrees of freedom (df). The calculated chi-squire values were compared with the tabulated chi-square values to specify the level of relationship or association between variables and ($P<0.05$) regarded as significant, minitab version 16 was used for this study

Prevalence was calculated using the formula:

$$\text{Prevalence} = \frac{\textit{Number of animals positive} \times 100}{\textit{Total animals tested}}$$

CHAPTER FOUR

RESULTS

4.1 Prevalence of Bovine Tuberculosis Infection in Borno and Yobe States, based on Caudal Fold Intradermal Tuberculin Test

The result of tuberculin test conducted on the 109 herds of cattle within the study area is presented in (Table 4.1). A total of 6,293 cattle were tested for bTB infection in Borno and Yobe States. Out of the total tested, 645 were positive and 5504 were negative giving a prevalence rate of 10.3%. In Borno State 3290 cattle were tested, out of which 348 were positive with a prevalence rate of 10.6%. In Yobe State 3003 cattle were tested out of which 279 were positive with a prevalence rate of 9.9%. The chi-square (χ^2) test of significance based on the result between the two states showed that the difference was not statistically significant (P>0.05).

4.1.1 Prevalence of bovine TB infection in settled and semi-settled herds based on caudal fold intradermal tuberculin test

The prevalence of *M. bovis* infection in cattle based on caudal fold Intradermal Tuberculin Test in Settled and Semi-Settled herds system of husbandry is presented in (Table 4.2). For the two states, out of the 6293 cattle tested, 4237 were from settled herds while the remaining 2056 were from the semi-settled herds system of managment. Out of cattle tested in the settled herds, 333 were positive with a prevalence rate of 10.3%. For the semi-settled herds 212 out of the 2056 tested were positive with a prevalence rate of 10.2%. The chi-square test of significance between the prevalence of *M. bovis* infection for the two management systems shows that the difference was not statistically significant (P>0.05).

Table 4.1: Prevalence of bovine TB infection in Borno and Yobe States, based on caudal fold intradermal tuberculin test

State	№ of Cattle tested(%)	Positive(%)	Negative(%)	Prevalence	(P-Value)
Borno	3290(52.3)	348(54.0)	2942(51.9)	10.6%	0.673
Yobe	3003(47.7)	297(46.0)	2706(48.1)	9.9%	
Total	**6293**	**645**	**5648**	**10.3%**	

(χ^2 = with 2df = 1.84, P<0.673)

(P<0.05) regarded as significant

Table 4.2: Prevalence of bovine TB infection in settled and semi-settled cattle herds, based on caudal fold intradermal tuberculin test

Management	№ of Cattle tested(%)	Positive(%)	Negative(%)	Prevalence	(P-Value)
Settled	4237(67.3)	433(67.1)	3804(67.5)	10.2%	0.775
Semi-Settled	2056(32.7)	212(32.9)	1844(32.5)	10.3%	
Total	**6293**	**645**	**5648**	**10.3%**	

(χ^2 = with 2df = 1.24, P<0.775)

(P<0.05) regarded as significant

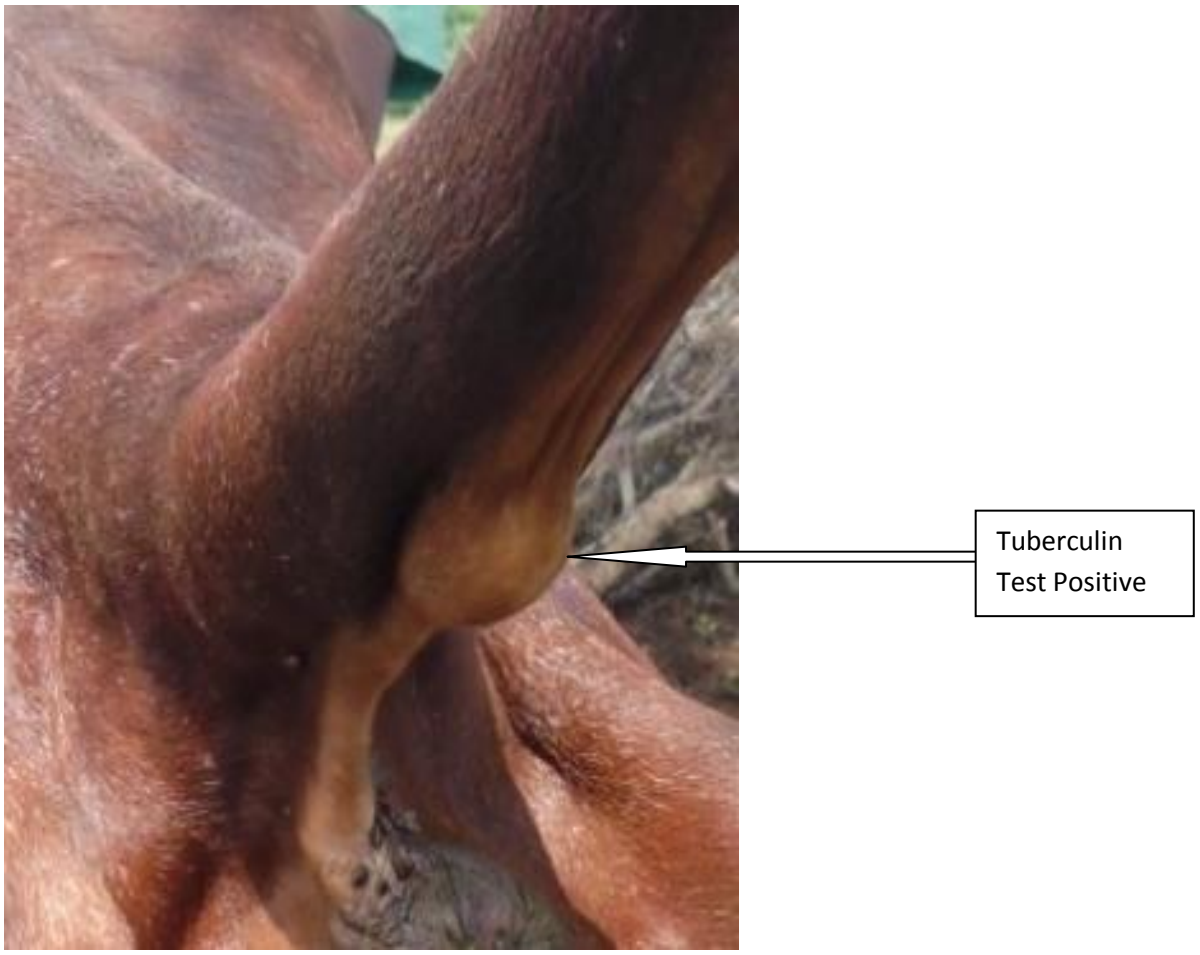


Plate V: Showing tuberculin test positive result

4.1.2 Prevalence of bovine TB infection in male and female cattle based on caudal fold intradermal tuberculin test

Prevalence of *M. bovis* infection based on sex of cattle tested is presented in (Table 4.3). A total of 6293 cattle, consisting 1902 males and 4391 females were tested. Of the males tested 183 were positive with a prevalence rate of 9.6%, while on the other hand out of the female cattle tested, 462 were positive with a prevalence rate of 10.5%. The chi-squire (χ^2) test of significance based on sex showed the difference was not statistically significant (P>0.05).

4.1.3 Prevalence of bovine TB infection among major breeds of cattle tested based on caudal fold intradermal tuberculin test

Four major breeds of cattle tested in the two states were Bunaji (White Fulani), Rahaji (Red Bororo), Wadara and Sokoto Gudali. The prevalence of bTB infection among major breeds of cattle tested in the two states is presented in (Table 4.4). Out of the 6293 different breeds of cattle tested 2414, 1993, 1387 and 501 were from Bunaji, Rahaji, Wadara, and Sokoto Gudali breeds of cattle respectively. For the Bunaji breed tested, 267 were found to be positive with a prevalence of 11.1%, for the Rahaji breed, 253 were positive with a prevalence rate of 12.7%, for the Wadara breed 112 were positive with a prevalence rate of 8.1% and for the Sokoto Gudali breed 13 were positive with a prevalence rate of 2.5%. The chi-squire (χ^2) test of significance based on major breeds tested shows the difference was statistically significant (P<0.05).

Table 4.3: Prevalence of bovine TB infection in males and females cattle tested based on caudal fold intradermal tuberculin test

Sex	№ of Cattle tested(%)	Positive(%)	Negative(%)	Prevalence	(P-Value)
Male	1902(30.2)	183(29.9)	1719(30.7)	9.6%	0.53
Female	4391(69.8)	462(70.1)	3919(69.3)	10.5%	
Total	**6293**	**645**	**5648**	**10.3%**	

(χ^2 = with 2df = 1.04, P<0.53)

(P<0.05) regarded as significant

Table 4.4: Prevalence of bovine TB infection among major breeds of cattle tested, based on caudal fold intradermal tuberculin test

Breed	№ of Cattle tested(%)	Positive(%)	Negative(%)	Prevalence	(P-Value)
Bunaji	2414(38.4)	267(41.4)	2147(38.0)	11.1%	0.037
Rahaji	1993(31.7)	253(39.2)	1740(30.8)	12.7%	
Wadara	1387(22.0)	112(17.4)	1275(22.5)	8.1%	
Sokoto Gudali	501(8.0)	13(2.0)	486(8.8)	2.5%	
Total	**6293**	**645**	**5648**	**10.3%**	

(χ^2 = with 4df = 9.84, P<0.037)

(P<0.05) regarded as significant

4.1.4 Prevalence of bovine TB infection among different age groups of cattle tested, based on caudal fold intradermal tuberculin test

The ages of cattle tested were distributed in to four different groups (1-2yrs, >2 – 4yrs, >4 – 6yrs and>6yrs). The prevalence of bTB infection based on different age groups is presented in (Table 4.5). Out of the 6293 cattle tested, 1431, 1729, 1874 and 1259 were between the ages of 1-2yrs, >2 – 4yrs, >4 – 6yrs and >6yrs respectively. For age group 1 – 2yrs, 37 were positive with a prevalence rate of 2.6%, for those between >2 -4yrs 218 were positives with a prevalence rate of 12.6%, between >4 – 6yrs 273 were positives with a prevalence rate of 14.6% and greater than 6yrs 117 were positives with a prevalence rate of 9.3%. The chi-squire (χ^2) test of significance between the age groups of cattle tested showed the difference was statistically significant ($P<0.05$).

4.1.5 Prevalence of bovine TB infection in relation to body condition score of cattle tested based on caudal fold intradermal tuberculin test

Prevalence of bTB infection in relation to 3 different body condition scores (Good, Fair and Poor) is presented in (Table 4.6). Out of 6293 cattle tested for bTB infection within the study area, 1043, 3782 and 1468 were assessed to be in good, fair and poor body conditions respectively. For those that were in good body condition score, 91 were positive with a prevalence rate of 8.7%, for those that were in fair body condition score 374 were positive with a prevalence rate of 9.9%, and for those that were in poor body condition score 180 were positive with a prevalence rate of 12.3%. The chi-squire (χ^2) test of significance between body condition scores of cattle tested shows that there was a significant difference in prevalence of bTB ($P<0.05$).

Table 4.5: Prevalence of bovine TB infection among different age groups of cattle tested, based on caudal fold intradermal tuberculin test

Age	№ of Cattle tested(%)	Positive(%)	Negative(%)	Prevalence	(P-Value)
1 – 2yrs	1431(22.7)	37(5.7)	1394(25.1)	2.6%	(0.025)
>2 – 4yrs	1729(27.5)	218(33.8)	11511(26.6)	12.6%	
>4 – 6yrs	1874(29.8)	273(42.3)	1601(28.1)	14.6%	
>6yrs	1259(20.0)	117(18.1)	1142(20.3)	9.3%	
Total	**6293**	**645**	**5648**	**10.3%**	

(χ^2 = with 4df = 10.75, P<0.025)

(P<0.05) regarded as significant

Table 4.6: Prevalence of bovine TB infection in relation to body condition scores of cattle tested, based on caudal fold intradermal tuberculin test

BCS	№ of Cattle tested(%)	Positive(%)	Negative(%)	Prevalence	(P-Value)
Good	1043(16.6)	91(14.1)	952(16.9)	8.7%	(0.049)
Fair	3782(60.1)	374(58.0)	3408(60.2)	9.9%	
Poor	1468(23.3)	180(27.9)	1288(22.9)	12.3%	
Total	**6293**	**645**	**5648**	**10.3%**	

(χ^2 = with 2df = 8.53, P<0.049)

(P<0.05) regarded as significant

4.2 Prevalence of Bovine TB in Borno and Yobe States, based on TB-like Lesions in Slaughtered Cattle

The result of post-mortem inspection conducted on slaughtered cattle in 20 abattoirs/slaughter houses within the study area is presented in (Table 4.7). A total of 4,130 cattle were examined, out of which 379 were positive with a prevalence rate of 9.2%. In Borno State, a total of 2207 were examined out of which 212 were positive with a prevalence rate of 9.6%. On the other hand, in Yobe State a total of 1923 were examined, 167 were positive with a prevalence rate of 8.7%. The chi-square (χ^2) test of significance between the prevalence of bTB in the two states showed that there was no significant association between the two states (P>0.05).

4.2.1 Prevalence of bovine TB in males and females of cattle examined based on TB-like lesions in slaughtered cattle

The prevalence rate of bTB in male and female cattle examined is presented in (Table 4.8). A total of 4130 cattle, consisting 1794 males and 2336 females were examined for TB-like lesions. Out of the males examined 208 were positive with a prevalence rate of 9.5%. Out of the females examined 171 were positive with a prevalence rate of 8.9%. The chi-squire (χ^2) test of significance in the occurrence of bTB between males and females shows the difference was not statistically significant (P<0.05).

Table 4.7: Prevalence of bovine TB in Borno and Yobe states, based on TB-like lesions in slaughtered cattle

State	№ of Cattle Examined(%)	Positive(%)	Negative(%)	Prevalence	(P-Value)
Borno	2207(53.4)	212(55.9)	1995(53.2)	9.6%	0.572
Yobe	1923(46.6)	167(44.1)	1756(46.8)	8.7%	
Total	**4130**	**379**	**3751**	**9.2%**	

(χ^2 = with 2df = 2.14, P<0.572)

(P<0.05) regarded as significant

Table 4.8: Prevalence of bovine TB in males and females, based on TB-like lesions in slaughtered cattle

Sex	№ of Cattle Examined(%)	Positive(%)	Negative(%)	Prevalence	(P-Value)
Male	1794(43.4)	208(54.9)	1586(42.3)	9.5%	0.563
Female	2336(56.6)	171(45.1)	2165(57.7)	8.9%	
Total	**4130**	**379**	**3751**	**9.2%**	

(χ^2 = with 2df = 2.11, P<0.563)

(P<0.05) regarded as significant

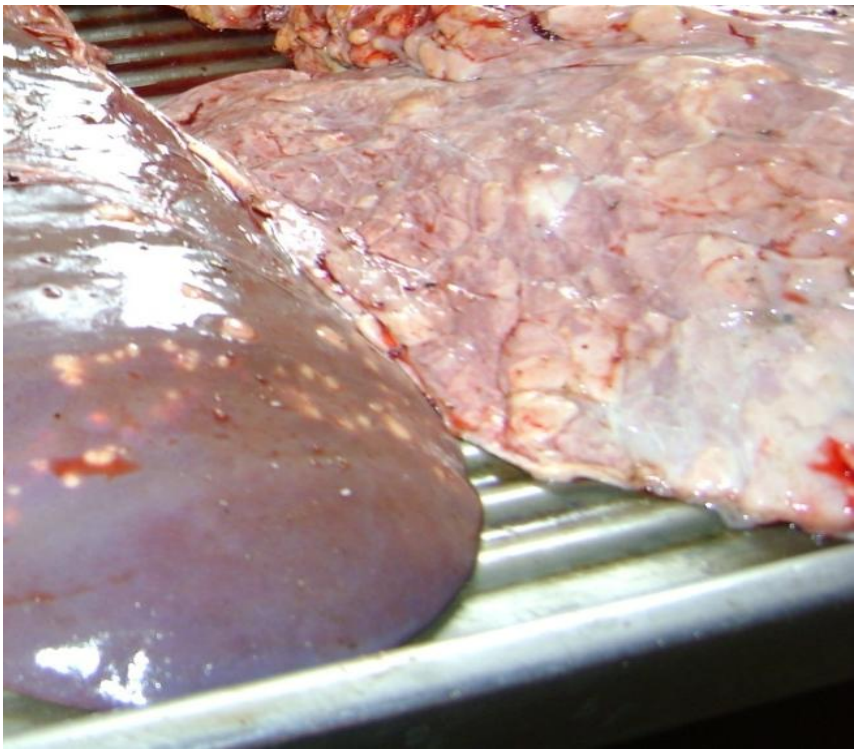


Plate VI: TB-like lesions from lung and liver tissues in slaughtered cattle

4.2.2 Prevalence of bovine TB among major breeds of cattle examined based on TB-like Lesions in Slaughtered Cattle

The Four major breeds of cattle examined in abattoir for TB-like lesions in the two states were Bunaji (White Fulani), Rahaji (Red Bororo), Wadara and SokotoGudali. The prevalence of bTB among the major breeds of cattle examined is presented in (Table 4.9). Out of the 4130 cattle examined 1881, 1051, 773 and 425 were of Bunaji, Rahaji, Wadara, and Sokoto Gudali breeds of cattle respectively. Out of the Bunaji cattle examined, 183 were found to be positive with a prevalence of 8.9%. For the Rahaji breed examined, 97 were positive with a prevalence rate of 9.4%. For the Wadara breed examined, 73 were positive with a prevalence rate of 9.2% while for the Sokoto Gudali breed examined 39 were positive with a prevalence rate of 9.6%. The chi-squire (χ^2) test of significance based on the four major breeds of cattle examined showed that the difference was not statistically significant ($P>0.05$).

4.2.3 Prevalence of bovine TB among different age groups based on TB-like Lesions in Slaughered Cattle

The prevalence of bTB among different age groups based on TB-like lesions in slaughtered cattle is presented in (Table 4.10). The ages of cattle examined were classified into three groups (≤4yrs, >4yrs – ≤6yrs and >6yrs). Out of 4130 slaughtered cattle examined at post-mortem for TB-like lesions, 154, 2468 and 1509 were between the ages of ≤4yrs, >4 – ≤6yrs and >6yrs respectively. In the age group ≤4yrs, 9 were positive giving a prevalence rate of 5.8%, in the group >4 and ≤6yrs 187 were positive with a prevalence rate of 7.5%, and for age group over 6yrs 183 were positive with a prevalence of 12.1%. The chi-squire (χ^2) test of significance was used to determine the association between the age groups of cattle and there was significant difference in the prevalence of bBT between the various age groups ($P<0.05$).

Table 4.9: Prevalence of bovine TB among major breeds of cattle examined, based on TB-like lesions in slaughtered cattle

Breed	№ of Cattle Examined(%)	Positive(%)	Negative (%)	Prevalence	(P-Value)
Bunaji	1881(45.5)	185(48.3)	1698(45.3)	9.8%	(0.139)
Rahaji	1051(25.4)	99(25.6)	954(25.4)	9.4%	
Wadara	773(18.7)	72(19.3)	701(18.7)	9.3%	
Sokoto Gudali	425(10.3)	36(10.3)	390(10.4)	8.4%	
Total	**4130**	**379**	**3751**	**9.2%**	

(χ^2 = with 4df = 3.54, P<0.139)

(P<0.05) regarded as significant

Table 4.10: Prevalence of bovine TB among different age groups of cattle examined, based on TB-like lesions in slaughtered cattle

Age	№ of Cattle Examined(%)	Positive(%)	Negative(%)	Prevalence	(P-Value)
≤4yrs	154(3.7)	9(2.9)	143(3.8)	5.8%	(0.015)
>4 – 6yrs	2467(59.7)	187(48.8)	2282(60.8)	7.5%	
>6yrs	1509(36.5)	183(48.3)	1326(35.4)	12.1%	
Total	**4130**	**379**	**3751**	**9.2%**	

(χ^2 = with 3df = 11.23, P<0.015)

(P<0.05) regarded as significant

4.2.4 Distribution of bovine TB lesions in different organs of slaughtered cattle

The distribution of suspected gross TB lesions in different organs of affected cattle shows that the lungs had the highest number of TB-like lesions with 237(62.5%), followed by lymph nodes with 103(27.2%) while liver, intestines, and spleen were 21(5.5%), 12(3.2%) and 6(1.6%) respectively (Table 4.11).

Table: 4.11: Distribution of bovine tuberculosis lesions in different organs of slaughtered cattle

Organs	Location of TB- lesions	Percentage (%)
Lungs	237	62.5
Lymph node	103	27.2
Liver	21	5.5
Intestine	12	3.2
Spleen	6	1.6
Total	**379**	**100**

4.3 Risk Factors Related to Cattle Handlers Awareness/Knowledge of Bovine TB

The results of the analysis of risk factors related to cattle handlers' awareness/knowledge of bTB is presented (Table 4.12).

Regarding awareness/knowledge of bTB based on sex; the results indicated that males had higher percentage of awareness/knowledge (87.3%) compared to females (48.3%). Awareness/knowledge of bTB based on age; individuals that were above 40yrs had higher percentage (82.9%) compared to those less than 40yrs (78.4%). On awareness/knowledge of bTB based on level of formal education; those handlers with tertiary level of education were more informed about bTB (96.6%) followed by those with secondary, primary and no formal education respectively (94.1%, 88.7% and 46.3%). For awareness/knowledge of bTB based on occupation of the respondents; the results showed that meat inspectors (100%) and butchers (98.1%) were more informed than cattle rearers, cattle marketers and milk sellers with 71.6%, 70.8% and 29.4% respectively.

4.3.1 Risk factors related to cattle handlers on the habit of drinking raw milk

The results of the analysis of risk factors related to cattle handlers on the habit of drinking raw milk are presented (Table 4.13).

Regarding on the habit of drinking raw milk based on sex; the results indicated that females had higher percentage (80.2%) compared to males (37.8%).

Habit of drinking raw milk based on age; individuals that were above 40 years had higher percentage (52.9%) compared to those that were less than 40 years (41.8%).

Based on level of formal education; it was found that those who had no formal education had higher percentage (61.8%) followed by those with primary, secondary and tertiary with 55.1%, 54.8% and 22.4% respectively.

Based on occupation of the respondents; It was higher among cattle rearers (80.2%) followed by milk sellers, cattle marketers, meat inspectors and butchers with 69.6%, 40.2%, 12.7% and 10.9%, respectively.

Table 4.12: Risk factors related to cattle handlers awareness/knowledge of bovine TB

Variable	Awareness/Knowledge
Sex	
Male	315/361(87.3%)
Female	57/118(48.3%)
Age (Years)	
≥ 40yrs	193/263(78.4%)
< 40yrs	179/216(82.9%)
Level of Formal Education	
None	74/160(46.3%)
Primary	125/141(88.7%)
Secondary	112/119(94.1%)
Tertiary	57/59(96.6%)
Occupation	
Cattle Rearers	97/137(70.8%)
Cattle Marketers	68/95(71.6%)
Milk Sellers	25/85(29.4%)
Butchers	105/107(98.1%)
Meat Inspectors	55/55(100%)

Table 4.13: Risk factors related to cattle handlers on the habit of drinking raw milk

Variable	Drinking of row Milk
Sex	
Male	122/341(37.8%)
Female	93/116(80.2%)
Age (Years)	
≥ 40yrs	105/251(41.8%)
< 40yrs	110/208(52.9%)
Level of Formal Education	
None	94/152(61.8%)
Primary	75/134(56.1%)
Secondary	63/115(54.8%)
Tertiary	13/58(22.4%)
Occupation	
Cattle Rearers	105/131(80.2%)
Cattle Marketers	37/92(40.2%)
Milk Sellers	55/79(69.6%)
Butchers	11/102(10.9%)
Meat Inspectors	7/55(12.7%)

4.3.2 Awareness/knowledge of cattle handlers to threat/risk of bovine TB transmission

Results showed that 66.3% (303 of 457) respondents were aware/knew that humans could contract diseases from animals, 55% (264 of 479) of respondents were aware/knew tuberculosis as a disease entity, 42.9% (183 of 427) of respondents were aware/knew that cattle also may have tuberculosis, 27.5% (113 of 411) and 20.4% (84 of 411) of respondents were aware/knew cattle could infect humans with TB and vice-versa, 25.4% (101 of 395) of respondents were aware/knew of two or three modes of TB transmission, 22.5% (89 of 395) respondents were aware/knew two or three clinical sign of TB in cattle and 54,4% (229 of 421) respondents were aware/knew that TB could be cured (Figure 4.1).

4.3.3 Attitudes/practices of cattle handlers to threat/risk of bovine TB transmission

The results showed that 87.3% (411 of 471) respondents routinely drank cow milk, and 45.6% (215 of 471) of respondents could drink unboiled/unpasteurized milk. Only 12.6% (41 of 325) of respondents had ever been tested for TB in their life time. Only 8.9% (29 of 325) of respondents remembered receiving BCG vaccine. 11.8% (49 of 417) of respondents used protective materials before handling cattle/carcasses and 13.8% (41 of 297) respondents washed hands before and after handling cattle and cattle products (Figure 4.2).

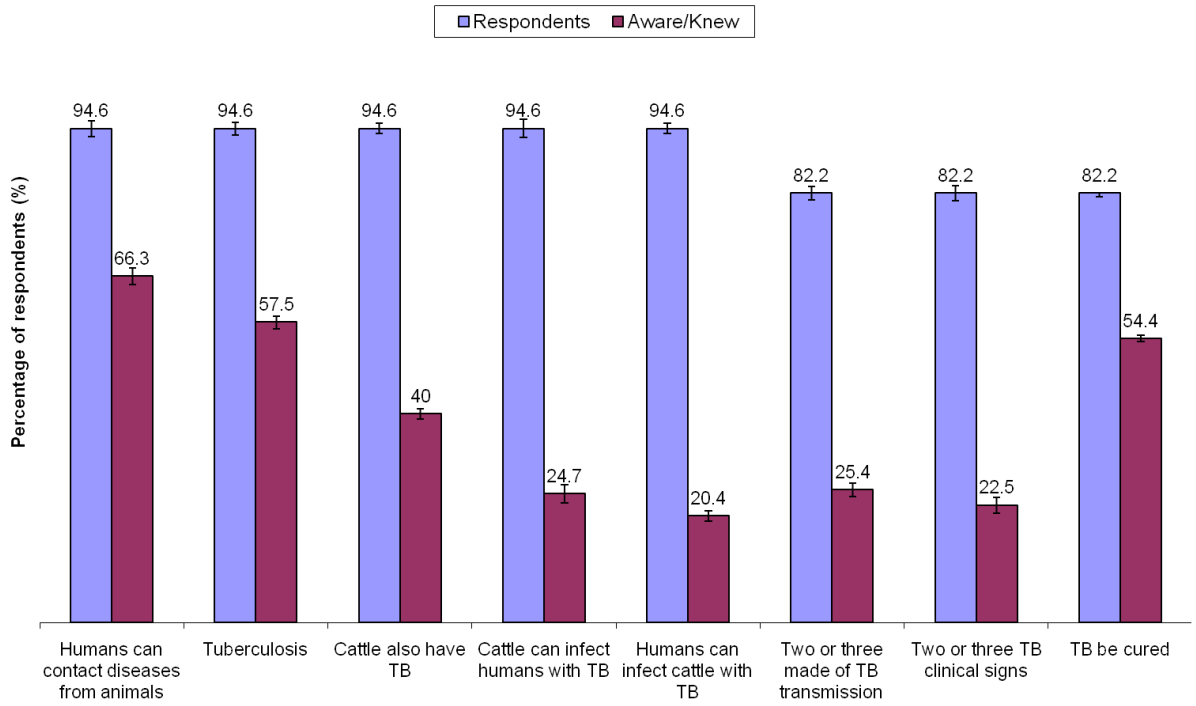


Figure 4.1: Awareness/Knowledge of cattle handlers to threat/risk of bovine TB transmission

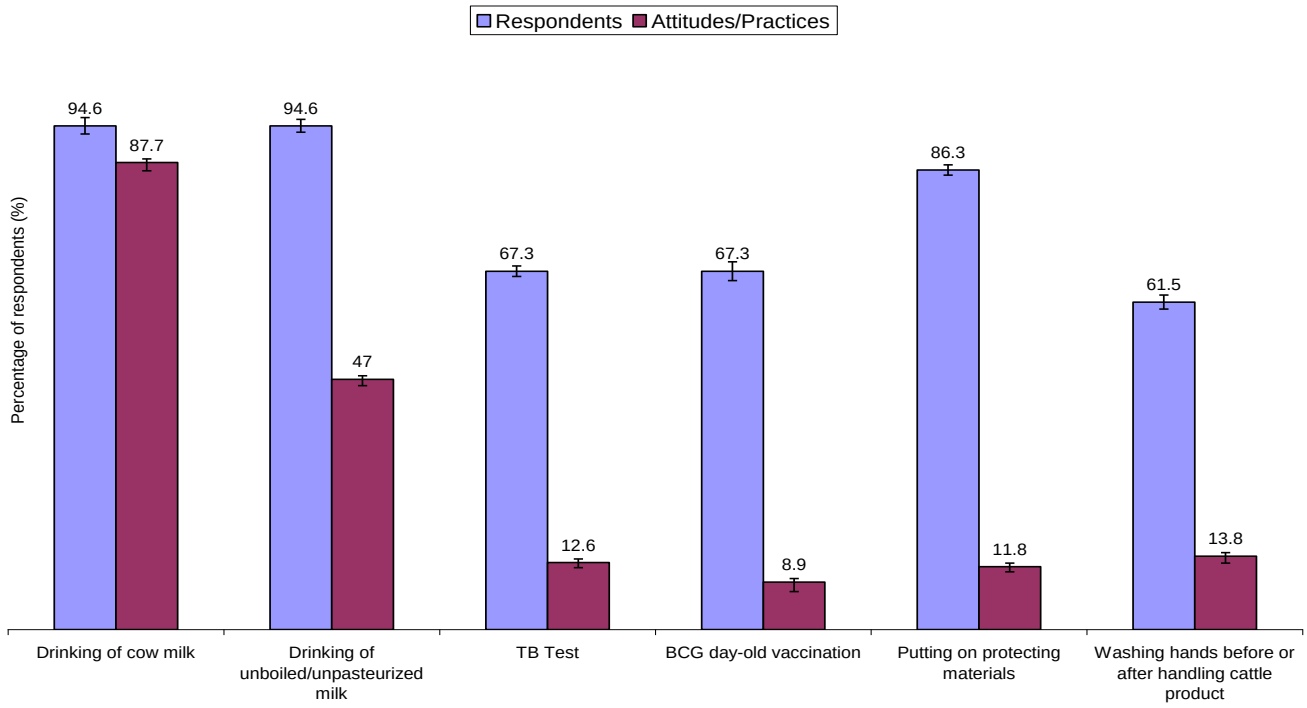


Figure 4.2: Attitudes/Practices of cattle handlers to threat/risk of bovine TB transmission

4.4 Characteristics of TB Patients in Borno and Yobe States

Characteristics of TB patients obtained from specimen submission forms are presented (Table 4.14). A total of 391 TB patients were collected, consisting of 217(55.5%) males and 174 (44.5%) females. Of the total TB patients, 148 (37.9%) have had BCG immunization while the rest were not sure of having been immunised.

On the history of contact with cattle (living, working or staying in a farm or occupational contact), 303 (77.5%) of the 391 patients had prolong close contact to cattle, 233 (59.6%) used to drink or consume raw milk and 17(4.3%) were also HIV positive.

4.4.1 Isolation and identification of tubercle bacilli from human sputa in Borno and Yobe States based on culture, acid-fast stainaing and TB Ag MPT64 (SD-bioline)

Results of the isolation and identification of tubercle bacilli from human sputa in Borno and Yobe States based on culture, acid- fast staining and SD-bioline are presented (Table 4.15). Out of the total samples processed (391), 94(24.0%) were culture positive, 67(71.3%) were acid-fast stain positive and 62(66.0%) were SD-bioline positive for *Mycobacterium tuberculosis* complex. In Borno State a total of 50(24.4%) were culture positive, 41(82.0%) were acid-fast stain positive and 37(74.0%) were SD-bioline positive. On the other hand, in Yobe State, a total of 44(23.7%) were culture positive, 26(59.1%) were acid-fast stain positive and 25(56.8%) were SD-bioline positive.

Table: 4.14: Characteristics of TB patients in Borno and Yobe States

Patient Characteristics	Reponses (%)
Sex	
- Males	217(55.5%)
- Females	174(44.5%)
Prolong close Contact with cattle	
- Yes	303(77.5%)
- No	88(22.5%)
Consumption of raw milk	
- Yes	233(59.6%)
- No	158(40.4%)
BCG in the past	
- Yes	148(37.9%)
- No	243(62.1%)
With and or without HIV	
- Yes	17(4.3%)
- No	374(95.7%)

Table 4.15: Isolation and identification of tubercle bacilli from human sputa in Borno and Yobe States, based on culture, acid-fast staining and TB Ag MPT64 (SD-bioline)

States	Sputum Samples	Culture Positive	Acid-fast stain Positive	SD-Bioline Positive
Borno	205	50(24.4%)	41(82.0%)	37(74.0%)
Yobe	186	44(23.7%)	26(59.1%)	25(56.8%)
Total	**391**	**94(24.0%)**	**67(71.3%)**	**62(66.0%)**

4.4.2 Isolation and identification of tubercle bacilli in humans male and female Sputa based on culture, acid-fast staining and TB Ag MPT64 (SD-bioline)

The isolation and identification of tubercle bacilli in humans male and female based on Culture, Acid-fast Stain and SD-bioline is presented (Table 4.16). Out of the 391 total samples processed, 61(28.1%) of the 217 males were culture positive, 43(70.5%) were acid-fast stain positive and 38(62.3%) were SD-bioline positive. Of the 174 females, 33(18.9%) were culture positive, 25(75.6%) acid-fast stain positive and 24(72.7%) SD-bioline positive.

4.4.3 Isolation and identification of tubercle bacilli from human sputa with and without history of BCG vaccination based on culture, acid-fast staining and TB Ag MPT64 (SD-bioline)

The isolation and identification of tubercle bacilli in humans with the history of BCG vaccination based on Culture, Acid-fast Stain and SD-bioline is presented (Table 4.17). Of the 391 samples processed, 148(37.9%) had history of BCG vaccination of which 24(16.2%) were culture positive, 20(83.3%) were Acid-fast positive, and 17(70.8%) were SD-bioline positive. Out of those 243(62.1%) who did not remember their history of BCG vaccination, 71(29.2%) were culture positive, 47(66.2%) were acid-fast positive and 45(63.4%) were SD-bioline positive.

4.4.4 Isolation and identification of tubercle bacilli in human sputa with and without history of drinking raw milk, based on culture, acid-fast staining and TB Ag MPT64 (SD-bioline)

The isolation and identification of tubercle bacilli in human sputa who drink and those who do not drink raw milk, based on Culture, Acid-fast Stain and SD Bioline is presented (Table 4.18). Of the 391samples processed, 233(59.6%) had history of consuming raw milk of which 57(24.5%) were culture positive, 38(66.7%) were Acid-fast positive and 35(61.4%) were SD-bioline positive. Of those 158(40.4%) who do not use to drink raw milk, 37(23.4%) were culture positive, 29(78.4%) were Acid-fast stain positive and 27(73.0%) were SD-bioline positive.

Table 4.16: Isolation and identification of tubercle bacilli in human male and female sputa, based on culture, acid-fast staining and TB Ag MPT64 (SD-bioline)

Sex	Sputum Samples	Culture Positive	Acid-fast stain Positive	SD-bioline Positive
Male	217	61(28.1%)	43(70.5%)	38(62.3%)
Female	174	33(18.9%)	25(75.6%)	24(72.7%)
Total	**391**	**94(24.0%)**	**67(71.3%)**	**62(66.0%)**

Table 4.17: Isolation and identification of tubercle bacilli from human sputa with and without history of BCG vaccination based on culture, acid-fast staining and TB Ag MPT64 SD-bioline

BCG	Sputum Samples	Culture Positive	Acid-fast stain Positive	SD-bioline positive
Yes	148	24(16.2%)	20(83.3%)	17(70.8%)
No	243	71(29.2%)	47(66.2%)	45(63.4%)
Total	**391**	**94(24.0%)**	**67(71.3%)**	**62(66.0)**

Table 4.18: Isolation and identification of tubercle bacilli in humans sputa with and without history of drinking raw milk, based on culture, acid-fast staining and TB Ag MPT64 (SD-bioline)

DRM	Sputa	Culture Positive	Acid-fast stain Positive	SD-bioline Positive
Yes	233	57(24.5%)	38(66.7%)	35(61.4%)
No	158	37(23.4%)	29(78.4%)	27(73.0%)
Total	**391**	**94(24.0%)**	**67(71.3%)**	**62(66.0%)**

4.5 Isolation and Identification of Tubercle bacilli from Bovine Tissues in Borno and Yobe States based on Culture, Acid-fast Staining and TB Ag MPT64 (SD-bioline)

Results of isolation and identification of tubercle bacilli from bovine tissue samples obtained from major abattoirs and slaughter houses in Borno and Yobe States based on culture, acid-fast staining and SD-bioline are presented (Table 4.19). Samples were also obtained from cattle that were tuberculin test positive that were purchased, slaughtered, examined/inspected for suspected TB lesions. Out of the total 379 samples obtained, transported and kept frozen at the TB Laboratory in the Department of Veterinary Medicine, ABU, Zaria for almost 3years waiting to be processed, only 84 were processed while the rest were discarded because they were considered spoiled due to electrical power instability. Of the total processed, 16(19.0%) were culture positive, 15(93.8%) were acid-fast stain positive and 2(12.5%) were SD-bioline positive.

From Borno State, a total of 10(19.6%) were culture positive, 9(90.0%) were acid-fast stain positive and 1(10.0%) were SD-bioline positive. From Yobe State a total of 6(18.2%) were culture positive, 6(100%) were acid-fast stain positive and 1(16.7%) were SD-bioline positive.

4.5.1 Isolation of *Mycobacterium* from milk samples

No growth of Mycobacteria was observed on Lowenstain-Jensen medium from 8 milk samples collected from the tuberculin test positive cows. This could be probably due to long storage of the milk samples.

Table 4.19: Isolation and identification of tubercle bacilli in bovine tissues from Borno and Yobe States, based on culture, acid-fast staining and TB Ag MPT64 (SD-bioline)

States	Tessue Samples	Culture Positive	Acid-fast stain Positive	SD-bioline Positive
Borno	51	10(19.6%)	9(90.0%)	1(10.0%)
Yobe	33	6(18.2%)	6(100%)	1(16.7%)
Total	**84**	**16(19.0%)**	**16(93.8%)**	**2(12.5%)**

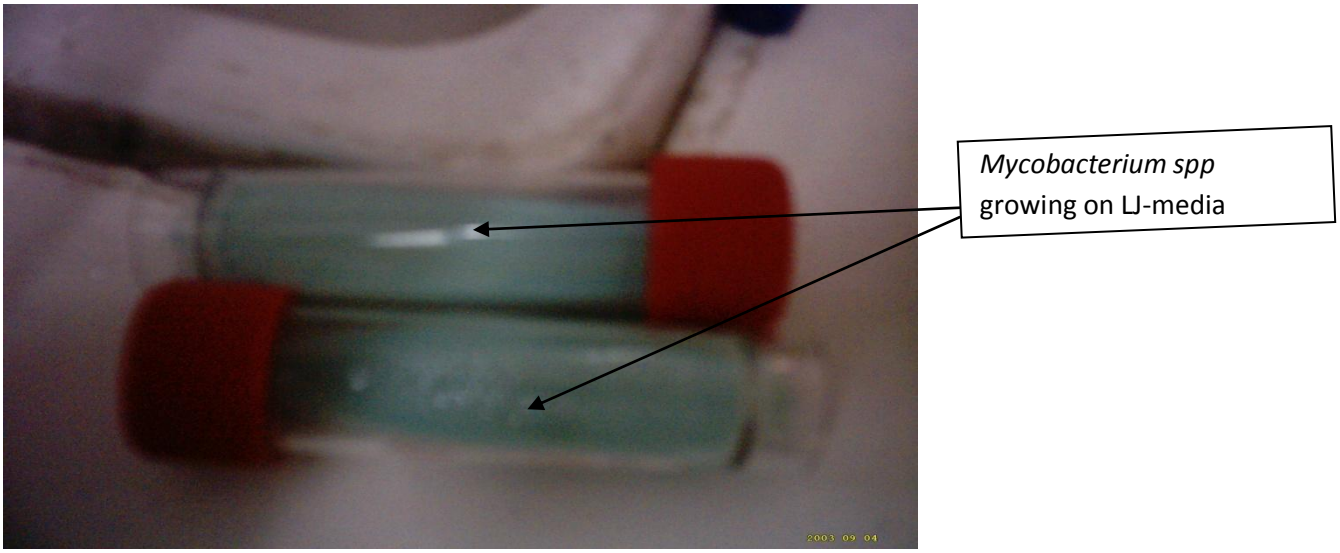


PLATE VII: *Mycobacteria spp* growing on LJ-media (Arrowed)

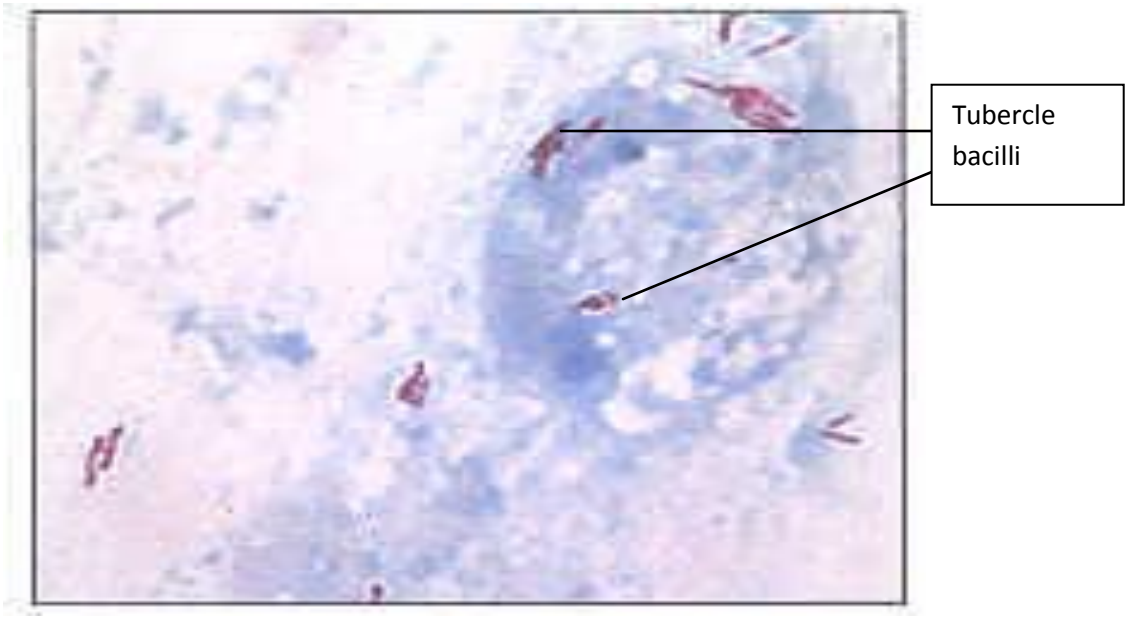


Plate VIII: *Mycobacteria spp* (acid-fast positive) under the microscope (×100)

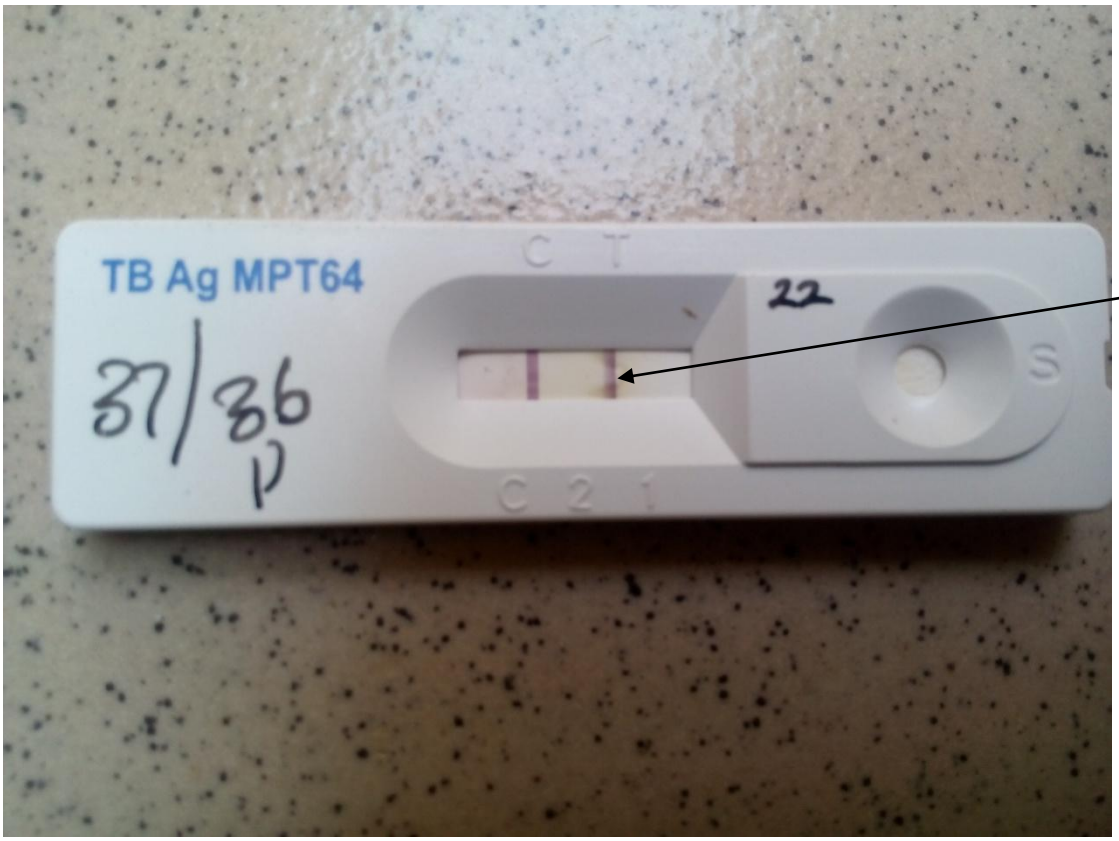


Plate IX: SD-bioline Ag MPT64, indicating positive result for *Mycobacterium tuberculosis* complex.

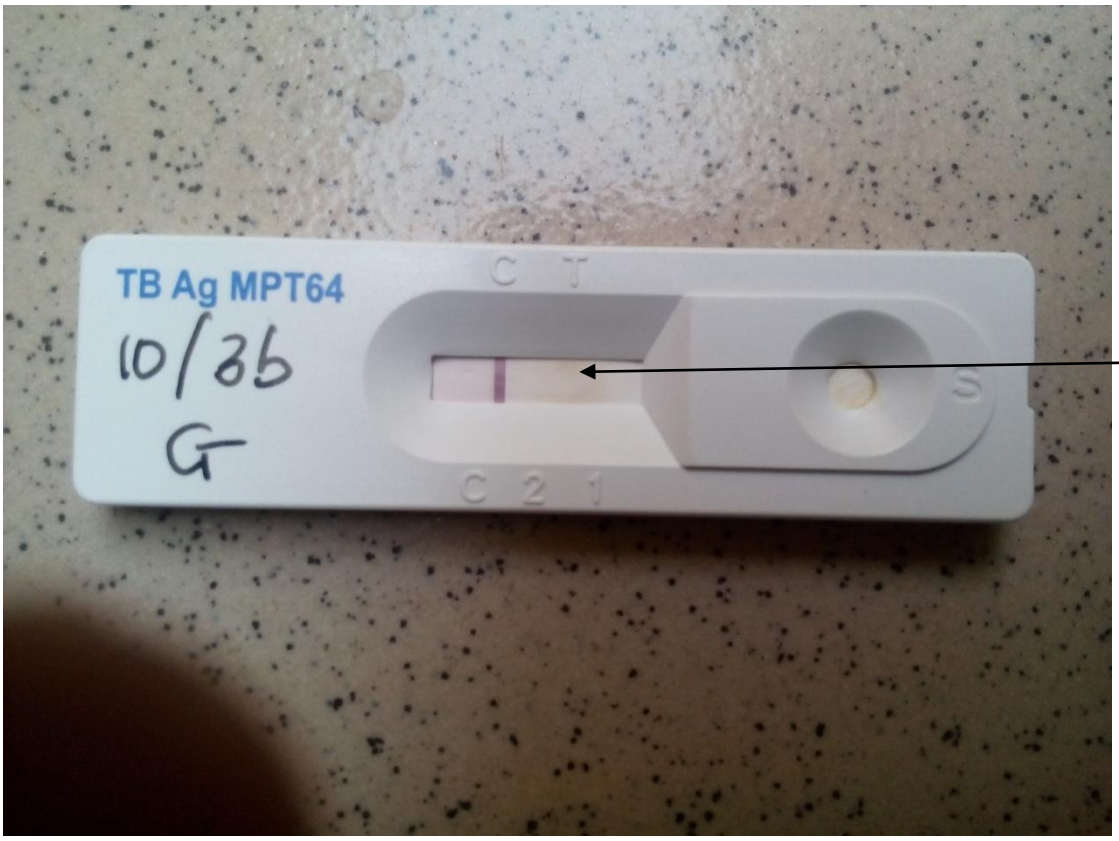


Plate X: SD-bioline Ag MPT64, indicating negative result for *Mycobacterium tuberculosis* complex.

4.6 Identification of Tubercle Bacilli from Bovine Tissues and Human Sputa Isolates Using Genotype MTBC Assay

Out of the 63 isolates identified by genotype MTBC Assay, 15(23.8%) were *M. bovis*, 6(9.5%) were *M. africanum* and 42(66.7%) were *M. tuberculosis.* However, it is worth noting that 4 *M. bovis* isolates were traced to human sputum while the remaining 11 were traced to bovine tissues. One *M. africanum* isolate was traced to bovine tissue while the remaining 5 were traced to human sputum. Two *M. tuberculosis* isolates were traced to bovine tissues while the remaining 40 were traced to human sputum (Table 4.19).

Table 4.20: Identification of tubercle bacilli from bovine tissues and human sputa isolates using genotype MTBC assay

Source	*M. bovis*	*M. tb*	*M. africanum*	Total
Bovine tissue	11	2	1	**14**
Human sputum	4	40	5	**49**
Total	**15**	**42**	**6**	**63**

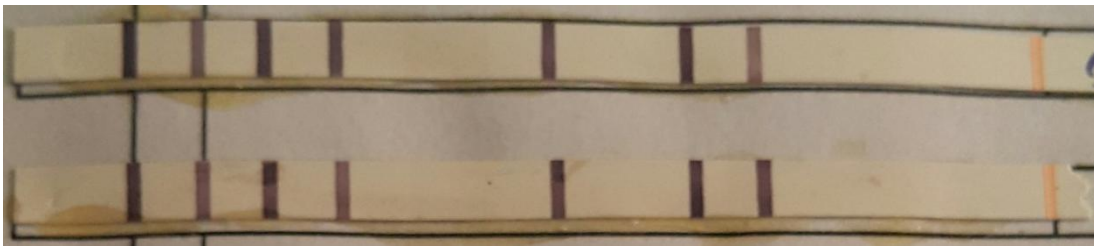


Plate XI: Genotype MTBC strips indicating *M. bovis*

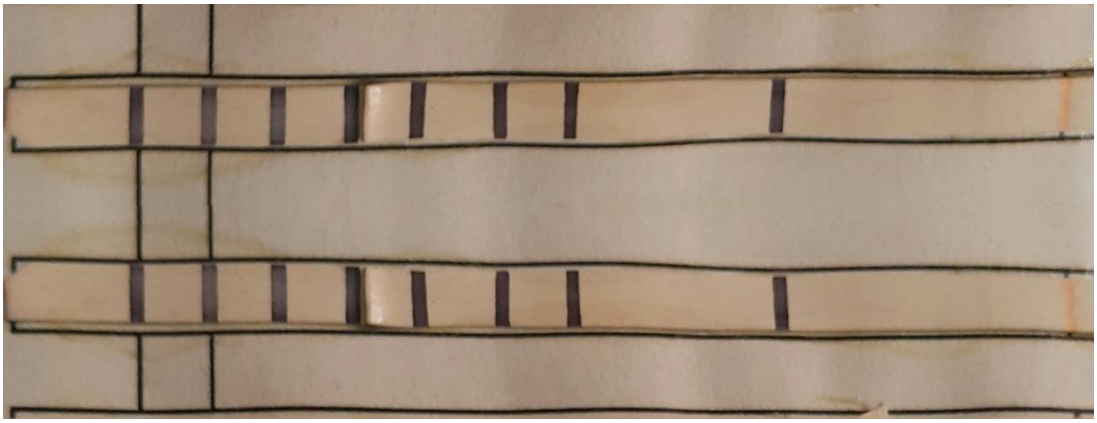


Plate XII: Genotype MTBC strips indicating *M. africanum*



Plate XIII: Genotype MTBC strips indicating *M. tuberculosis*

CHAPTER FIVE

DISCUSSION

In recent years, there has been an increasing concern on the threat of *Mycobacterium* species to public health, especially in developing countries like Nigeria, their association with the HIV/AIDS pandemic as reported elsewhere around the world which is making the situation worse (Gambo *et al*., 2013). This study described an epidemiologic study on (bTB) in two states of the North Eastern zone of Nigeria. The single caudal fold intradermal tuberculin test was used for the *invivo* diagnosis of the disease in cattle. This test procedure was found under the open range cattle husbandry system of management to be adequate for screening as well as routine testing using bovine PPD. Similar observations have been made by Eid (1975) and Alhaji (1976) where fewer problems with suspects or non-specific reactors were reported compared to single cervical comparative intradermal tuberculin test. The single caudal fold intradermal tuberculin test has been used extensively and successfully for decade in countries like United States of America (USA), Canada, Australia and New Zeeland. The results obtained in this study, showing an overall 10.3% prevalence of bTB infection is of great epidemiological and public health significance. This is because it has been documented that humans can contract the disease from their cattle as a result of close association (Cosivi, 1998). This is a major problem especially where cultural practices exist; as exemplified by the Fulani herdsmen, who live their entire lives with their animals, offering ample opportunity for zoonotic transmission of the infection. Also, bTB infection has been recognized as potential occupational risk for farm and abattoir workers (O'Reiley *et al.*, 1995). The results obtained from this study is slightly lower than the 14% recorded in a similar study conducted by Abubakar, (2007) but does not differ much from other studies conducted in other parts of the country (Shehu, 1988; Du-Sai *et al.*, 1994; Cadmus *et al.*, 2004). This indicates that bovine tuberculosis may be prevalent in all parts of Nigeria. The

reason for widespread distribution of infection might be attributed to management practices such as migration of nomadic herds from the north to the southern zones in search of greener pasture during the dry seasons and the consequent build up of Mycobacteria in the environment.

The (10.3%) prevalence of bTB infection observed in this study and the results of other studies conducted in Nigeria have indicated a steady presence of the infection over the last 39 years (1976 to 2015). For instance, Alhaji (1976) reported a 2.5% prevalence rate in northern region of Nigeria while in the same year Eid (1976) reported a 1.5% prevalence for cattle in the north western part of Nigeria. Similarly, Ayanwale (1984) reported 7.8% in a study in some southern states of Nigeria, Shehu (1988) reported 11.8% in Kaduna State while Abubakar (2007) reported 14% prevalence in Kaduna and FCT. Other such studies both in Nigeria and other African countries have shown a similar trend over the years (Ellwood, 1975; Abubakar, 1994; Du-Sai *et al.,* 1994; Jiwa *et al.,* 1997; Vekemans *et al.,* 1999; Shirima *et al.,* 2003; Cadmus *et al.,* 2004; Abubakar, 2010). This might not be unrelated to the lack of, or inadequate implementation of control policy in the region mainly due to socio-economic reasons. Added to the high costs of sustainable control programme are problems of social unrest due to political instability and ethnic wars especially among the local farmers and the Fulani herdsmen. It could also be as a result of intracontinental cattle movement, due to lack of proper border control arising from insufficient collaboration with bordering countries and the smuggling of live animals and hence lack of quarantine (Abubakar *et al.,* 2011).

Although management systems have been said to influence the prevalence of bTB infection among cattle population where the infection was found to be more prevalent among intensively managed

cattle than semi-intensive cattle population (O'Reiley *et al.,* 1995; Shirima 2003; Kempf *et al.,* 2005), this study did not show any significant effect of the management system on the prevalence of bTB infection. A similar observation was made in a study conducted by Shehu (1988), where a 13% prevalence reactor rate was reported among 315 heads of cattle tested in the nomadic herds and 10% among 155 heads of cattle in the semi-intensive (semi-nomadic) herds. This might be explained by the fact that cattle under the nomadic system are always on the move and are more likely to be exposed to cattle of unknown health status during grazing and also at watering points, particularly during the dry season. It could also be as a result of the animals grazing on contaminated pasture during movement and migration. However, this study has shown that bovine tuberculosis occur in both settled and semi-settled pastoral farming systems and this means that the cultural habit of pastoralists living indoors with their animals may be a significant risk factor for zoonotic transmision. It might also be a potential danger to wildlife and other animals which are susceptible to *Mycobacteria* species.

No significant relationship was found between sexes at ($P<0.05$). This finding is in agreement with previous studies conducted in other parts of Africa (Awah-Ndukum *et al.*, 2005; Cleaveland *et al.,* 2007). Indicating that bTB infection does not probably discriminate between sexes, although more females were tested during this study similar finding was reported by (Abubakar, 2007).

The difference in tuberculin test results observed among the four major breeds of cattle tested in this study is in agreement with similar observations made in other parts of Nigeria and Africa (Alhaji, 1976; Alemayahu *et al.,* 2010). There have been reports that the Bunaji breed (Zebu) is perhaps the most tuberculosis-resistant breed in Nigeria and Africa (Alhaji, 1976; Awah-Ndukum *et al.*, 2005), this was confirmed in this study, where Bunaji breed of cattle represented 38.4% of total cattle tested, but accounted for only 11.1% prevalence. Where as, Rahaji breed of cattle representing

31.7% of total cattle tested accounted for 12.7% prevalence. These have also been reported by (Cadmus *et al.,* 2004).

This study has shown that prevalence of bovine TB increased with age; up to 4-6years of age, after which it declines. This is similar to the findings by other researchers (Asseged *et al.*, 2000; Ameni et al., 2003). It is possible that the infection may not become established in young animals, but as they grow older, their chances of acquiring infection also increase due to the increased time of exposure while the decline at older age is explained by Tizard (2000) who stated that the lower prevalence to intradermal tuberculin test response in older animals is due to the immunodepression occurring during old age or poor body condition. A similar observation was also made in a study conducted by Shehu (1988) where he reported 20.9% tuberculin positive reactors among middle and old age group cattle. This finding has both epidemiological and public health importance because Fulani herders normally sell middle and very old cattle to other livestock farmers for fattening and these are later sold for slaughter. The implication of this is that it could facilitate the spread of the disease to other herds both at the cattle market and also when introduced into the new herd. If sold for slaughter, the risk of transmission from infected carcass to meat handlers, which has been documented is possible especially where butchers and meat inspectors process and inspect meat and meat products with minimal protective clothing and process offals from diseased carcasses with bare hands.

The high prevalence of bTB infection recorded in cattle with poor body condition scores compared to those with fair and good body condition scores is in agreement with previous reports (Asseged *et al.*, 2000; Ameni *et al.*, 2003). Poor body condition offers opportunity for

transmission of bTB infection and development of the disease and subsequently contributing to the variations in the prevalence of the disease among cattle with different body condition scores.

The prevalence of 9.2% from TB-like lesions among slaughtered cattle in an area where nearly a similar finding (10.3%) was made among live animals through tuberculin test and where these cattle are the main source of animals for slaughter, have serious epidemiological and public health implications. This becomes even more serious when lymph nodes and other visceral organs samples collected from these slaughtered animals indicated high number of acid-fast positive bacilli. This finding indicates that there is a relationship between prevalence of suspected bTB among slaughtered cattle and the prevalence of bTB infection among live cattle. This poses great danger of contracting the disease by the public especially abattoir workers and herdsmen. It further confirms that control measures are either not in place or inadequately applied; because in countries where control of bovine TB is in place, detection of lesions at the abattoir during meat inspection is usually very minimal (Cousins, 2001). This finding, also support earlier suggestion that, abattoir monitoring could be an essential element in the national bovine tuberculosis campaign and the most effective means of detecting residual infection in herds especially in countries that have achieved control of the disease. The association between the disease and sex of cattle examined for gross TB lesions was not statistically significant at ($P<0.05$). This is in agreement with previous studies in Africa and Nigeria (Omer *et al.,* 2001; Asseged *et al.*, 2004; Teklu *et al.*, 2004; Cleaveland *et al*., 2007; Abubakar *et al.*, 2012).

The preponderance of bTB cases in older cattle, as observed in this study, is in agreement with previous findings (Bonsu *et al.*, 2000; Cleaveland *et al.*, 2007; Abubakar *et al.,* 2012) and may partly

explain the importance of longetivity and prolonged exposure to the pathogen, added to the possible reactivation of latent infections in old and stressed animals (Cleaveland *et al.,* 2007).

Most of the observed TB-like lesions for bBT were from the lungs. This agrees with earlier studies where it was found that 70-90% of TB suspected lesions were found in the lungs and lymph nodes of the head or thoraxic cavity (Lepper, 1973; Neill *et al*., 1994; Palmer *et al*., 2002a; Philips, *et al.*, 2003; Abubakr, 2007). This may suggest that the most common route of transmission was through aerosol.

To the best of our knowledged, this is the first study focusing on level of awareness/knowledge of cattle handlers about bTB in Borno and Yobe States, North Eastern part of Nigeria. Cattle handlers with low level of education were most at risk of exposure to zoonotic bTB. Drinking of raw milk was common in the study area, but it was higher in females, milk sellers and cattle rearers where poverty levels are higher, literacy levels were lower and contact with livestock was higher. Approximately 85% of cattle and 82% of human populations in Africa have been estimated to live in areas where animal TB is either partly controlled or uncontrolled (Ayele *et al.,* 2004; Shitaye *et al.,* 2006). Isolation and identification of *M. bovis* from patients with pulmonary TB has been reported in Nigeria, Cameroon, Democratic Republic of Congo, Egypt and Tanzania (Cosivi *et al.,* 1998; Awah Ndukum *et al.,* 2010). The transboundary transmission of bTB in Africa and the threat of zoonotic TB due to *M. bovis* are very real (Awah-Ndukum *et al.,* 2010)

High human tuberculosis was observed among TB patients based on culture, acid-fast staining and SD-bioline from the same region where a similar observation was also made among cattle

population. However, it should be noted that this study was conducted among patients who were already suspected of having TB on the basis of symptoms and not from the open population. Nonetheless, this finding does not differ much from the 23.3% culture-positive result recorded by Abubakar (2007) in Kaduna and Federal Capital Territory, Abuja and 30% smear positive recorded by Lawson (2006) in a similar study in the Federal Capital Territory, Abuja among 1185 patients. The 17.1% sputum smear positive recorded in this study does not differ much from that reported in the National Tuberculosis and Leprosy Control Program case reports of 2003 and 2004 (Wim *et al.* 2004; NTBLCP 2010). Since specimen culture is not routinely used as a diagnostic tool in Nigeria as in most economically constrained countries, not many reports of TB through specimen culture are available. However, with the recent increase in the prevalence of TB worldwide especially in Africa and Asia, which is mainly as a result of the high prevalence of HIV/Aids in these regions (Umeh *et al.,* 2007), this finding is not surprising.

The TB/HIV co-infection observed in this study (4.3%) is lower than the 14.2% rate among tuberculosis patients reported in the National Drug-resistant tuberculosis prevalence survey of 2012 (Umeh *et al.*, 2007) and 8% reported by the United States Embassy in Nigeria (Nigeria Tuberculosis Fact Sheet, 2012). The low TB/HIV co-infection rate in this study could be as a result of the well established fact that most TB/HIV co-infection patients are not demonstrating tubercle bacilli with acid-fast staining. However, the TB/HIV co-infection rate in this study is close to what was reported (5.1%) before by (Nwachukwu *et al.*, 2009).

The difference in isolation and identification of tubercle bacilli between males and females TB patients observed in this study was in agreement with previous authors (Holmes *et al.,* 1998; Borgdorff *et al.,* 2000; Lawson, 2006; Abubakar, 2007). This could be argued that males might be

more at risk of having TB than females due to the nature of their work which is mainly out doors, therefore exposing them to more risks especially in Northern Nigeria, but women are mostly at home unemployed and thereby hardly exposed to risk of the infection. However, Nwachokor (2000) reported a high prevalence in females in a 30 year review of tuberculosis in Ibadan.

The study also shows that the rate of isolated and identified tubercle bacilli among those who can remember been vaccinated with BCG vaccine in the past was lower than among those who can not remember been immunized in the past. The high difference observed in this study would suggest that BCG immunization which is currently employed in Nigeria; as in most developing countries as the first vaccine given in the routine vaccination schedule of the National Programme on Immunizations (NPI) should continue to run concurrently with other TB control measures. Although, the significance of BCG has been questioned, this study shows that BCG vaccine seems to be protective, as an efficient method of prevention of TB especially in countries with high disease burden (Smith, 1982; Collins, 2001), but it is still a subject of continuous debate.

Although, no significant difference in the isolation and identification of tubercle bacilli rate observed between those who consume raw milk and those who do not and also no tubercle bacilli was isolated from milk samples obtained from tuberculin positive cattle, pasteurization of milk should not be ignored. The inability to isolate the organism from the milk samples could be due to prolong storage under refrigeration in the face of frequent electreicity outage. However, this finding was in support of Teklu *et al.*, (2004) who suggested that TB in cattle is principally transmited through respiratory discharges, but approximately only 1% of the tuberculous cows excrete tubercle bacilli in their milk. In a study performed in Tanzania, of 805 milk samples, *M. bovis* was isolated and confirmed in only 2 milk specimens (Kazwala *et*

al., 1998). The study might have also highlighted the necessity to trace other sources of bTB infection in humans in more detail other than milk.

The isolation of *M. tuberculosis* from cattle in this study confirmed similar findings of human-to-cattle transmission of *M. tuberculosis* by Ayele *et al.* (2004) and Abubakar (2007) It is generally held that disease in cattle due to *M. tuberculosis* is less severe than that caused by *M. bovis* (Francis 1958). However, the possibility of cross-contamination especially from infected animal handlers or milk hawkers to the cattle cannot be ruled out. This is also of public health importance as consumers of infected meat and milk stand the risk of getting infected.

Most importantly, the isolation of *M. bovis* from human sputum observed in this study is of significant and serious public health importance. This finding reveals that there is a definite association between animal infection and the disease in human. It further demonstrates the zoonotic importance of *M. bovis* in human infection and shows a typical animal-to-human transmission. This findings agrees with reports of Idigbe *et al.,* (1986), Cadmus *et al,* (2006), and Abubakar (2007) in Nigeria.

Similarly, the isolation of *M. africanum* species from both humans and cattle is of interest, as while it is virulent for cattle, it has only rarely been isolated from cattle (de Kantor *et al.,* 1979; Weber *et al.,* 1998). However, Cadmus *et al,* (2006) reported similar finding in a study in Ibadan, Nigeria. The identification of *M. africanum* in humans is of importance especially in immuno-compromised individuals.

The ability to accurately identify and trace species of *Mycobacterium* causing disease using molecular techniques has been identified as a powerful tool in investigating disease transmission within a population both in developed and developing countries (Cousins *et al.,* 1998). Therefore, molecular investigation of the isolates in this study has enhanced the understanding of the species involved in causing tuberculosis in both humans and cattle in the study area. However, this might justify saying that the Genotype MTBC seems to be more efficient, accurate and faster for field and epidemiological purposes as well as for conducting some PCR based molecular methods like the spoligotyping and VNTR which do not need high quality DNA and also can be conducted directly from clinical samples.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

Tuberculin test survey of 6,293 cattle involving 109 herds from 10 testing areas in two North Eastern States (Borno and Yobe) of Nigeria is described. A total of Six Hundred and Forty Five (645) cattle were positive with in a prevalence of 10.3%. The chi-square (χ^2) test of significance between the two states, between management systems and between sexes of cattle tested showed that the differences in prevalence were not statistically significant, but between 4 major breeds, age groups and body condition scores of cattle tested were significantly different ($P<0.05$).

Post-mortem inspection conducted in 20 abattoirs/slaughter houses in which 4,130 cattle were examined between March 2011 and November 2012 in Borno and Yobe States, out of which 379 were compatible with TB-like lesions with a prevalence of 9.2%. The chi-square (χ^2) test of significance between the two states, breeds and sex of cattle examined shows that the difference was not statistically significant while between age groups of cattle examined, there was a significant difference at ($P<0.05$).

Most cattle handlers were informed abaut bTB, its zoonotic nature and public health implications, but many of them were not informed about mode of transmission and clinical signs of the disease.

Isolates from bovine tissue samples obtained from major abattoirs and slaughter houses in Borno and Yobe States based on culture, acid- fast staining and SD-bioline procedures showed that 16(19.0%) were culture positive, 15(93.8%) were acid-fast stain positive and 2(12.5%) were SD-bioline positive.

Isolates from human sputums sampled obtained from human hospitals in Borno and Yobe States based on culture, acid- fast staining and SD-bioline procedures showed that 94(24.0%) were culture positive, 67(71.3%) were acid-fast stain positive and 62(66.0%) were SD-bioline positive.

Out of the 63 isolates identified by genotype MTBC Assay, 15(23.8%) were *M. bovis*, 6(9.5%) were *M. africanum* and 42(66.7%) were *M. tuberculosis.* However, it is worth noting that 4 *M. bovis* isolates were traced to human sputum while the remaining 11 were traced to bovine tissues. One *M. africanum* isolate was traced to bovine tissue while the remaining 5

were traced to human sputum. Two *M. tuberculosis* isolates were traced to bovine tissues while the remaining 40 were traced to human sputum.

6.2 Recommendations

1. There is urgent need for the incoperation of control of bovine tuberculosis into the National Tuberculosis Control programme to check the high prevalence of the disease in cattle and its subsequent consquences in humans. The colabration will reduce the human national incidence rate and mortality from tuberculosis observed in the country. For this to happen there must be cooperation between the Human Medical practitioners and the Veterinarians in the country. This is because; disease control programmes worldwide have begun to shift perspective from strictly one directional to multidisciplinary with the multi-sectorial collaborations that are required for successful control, particularly in developing countries.

2. Test and slaughter with adequate compensations paid to the owners by the governments should be implemented. This should be followed by rigorous meat inspection at abattoirs to help in detecting residual infections in herds. Also meat inspectors must carry out inspection using maximum protective clothing to protect them against infection. Abattoir monitoring is an essential element in a national bovine tuberculosis campaign to effectively detect residual infection in herds as is being used in other developed countries.

3. Furthermore, this study indicates that bTB plays a role in increasing the incidence and mortality from tuberculosis in humans. This means that protecting those working with cattle and ensuring microbiological safety of dairy products to the public would be an important

public health measure. This therefore calls for prompt implementation of control measures for bovine tuberculosis in the country to safeguard the human population and also to reduce economic losses in the livestock industry.

4. It is also important that the government should formulate a legislation concerning the sale of un-pasteurized milk to the public to reduce the risk of human infection from infected dairy products. In view of this, development of pasteurization points should be considered to pool milk from Fulani hawkers for pasteurization before selling to the public.

5. Culture or direct smear and molecular characterization of clinical isolates must be included as a diagnostic tool to ensure quick and accurate estimation of the actual burden of infection caused by various species of Mycobacterium.

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FORM II

FACULTY OF VETERINARY MEDICINE
AHMADU BELLO UNIVERSITY, ZARIA

NATIONAL BOVINE TB PROJECT

TUBERCULIN TEST RECORD
SINGLE CERVICAL COMPARATIVE TEST

Sate: _____ L/Govt.: _____

Town/Village: _____ Owner: _____

Type of Herd: _____ Species: _____

Kind of Herd: Mixed: _____ Dairy: _____ Beef: _____

Type of PPD: _____ Site of Test _____

Date of Test: _____ Hour of Test: _____

Date of Reading: _____ Hour of Reading: _____

Summary: Negative: _____ Suspect: _____ Reactor: _____ Total _____

Animal Identification	Age	Sex	Breed	Condition			Size of reaction in mm				Difference in mm		Reaction		Disposition
				G	F	P	Before		After						
							B	A	B	A	B	A	B	A	

APPENDIX II

FACULTY OF VETERINARY MEDICINE
AHMADU BELLO UNIVERSITY, ZARIA

NATIONAL BOVINE TB PROJECT
MYCOBACTERIOLOGY LABORATORY
SPECIMEN SUBMISSION FORM

Clinical No: _____
Pathology No: _____
Mycobacteriology Lab. No: _____

(1) **Sent by (Full name and address):**

_____
_____
_____

(2) **Patient/Code No.:**

(a) Full name and address: _____

_____
_____

(b) Sex: _____ Age: _____ Nationality: _____ Tribe: _____

(c) Occupation: _____

(d) History: _____

_____
_____
_____
_____

(3) **Specimen:**

(a) Date sent: _____

(b) Preservation: Frozen: _____ Formalin: _____ Others: _____

(c) Specimen Submitted:

Sputum [ ] Liver [ ]

Gastric lavage [ ] Spleen [ ]

Urine [ ] Others [ ]

Lymphnode (specify) [ ] _____

Lung [ ]

APPENDIX III

FACULTY OF VETERINARY MEDICINE
AHMADU BELLO UNIVERSITY, ZARIA

NATIONAL BAVINE TB PEROJECT
MYCOBACTERIOLOGY LABORATORY
SPEECIMENT SUBMISSION FORM

Clinical No.....

Mycobacteriology Lab.No.....

1.Sent by (Full name and address);

.....

2. Patient/Code No;.....

a). Full name and address.....

b). Sex..... Age.....Nationality.....Tribe..... ...

3. Occupation.....

4. Hitory

a). BCG vaccine in the past.....

b). Contact with animals.....

c. Consumption of lacally produced milk ''nono''.....

d. With or without HIV.....

5. Speciment;.....

a. With or without CPC.....

b. Date collected.....

APPENDIX IV

National Bovine TB Project

Dept. of Veterinary Surgery & Medicine,
Ahmadu Bello University, Zaria.

BOVINE TUBERCULOSIS IN CATTLE AND HUMANS IN NORTH-EAST ZONE, NIGERIA: RISK FACTORS ASSESSMENT

Questionnaire No.

State .....

L.G.A. .....

Community Location: - Urban .....Rural .....

Date .....

1

SECTION A: DEMOGRAPHIC AND SOCIOECONOMIC CHARACTERISTICS OF RESPONDENTS

QUESTION NO.	QUESTIONS	RESPONSE OPTIONS
A1	How old are you (years)?	
A2	What is your gender?	Male ..... Female .....
A3	What is your highest level of educational attainment?	Primary ..... Secondary ..... Tertiary ..... Quaranic---------- Others.....
A4	Where do you live?	Urban ..... Rural ..... Ruga----------
A5	How many of you on the average live together in a room?	.....
A6	How many adults? How many children?	Adults ..... Children .....
A7	How many windows does the room have?	.....
A8	How long have you been in contact with cattle?	.....
A9	When was the most recent outbreak of disease in your cattle?	..... None .....
A10	Describe the outbreak	
A11	Do Veterinary Doctors or animal health officers check your cattle?	Yes ..... No .....

SECTION B: **AWARENESS OF TUBERCULOSIS (TB)**

QUESTION NO.	QUESTIONS	RESPONSE OPTIONS
B1	Are you aware that humans can contract disease from cattle?	Yes ..... No ..... Don't know .....
B2	If yes, what type of disease can be contracted?	....
B3	If no to B2, why do you think that humans cannot contract disease from cattle?	
B4	Do you know of a disease called tuberculosis (Tarin fuka)?	Yes ..... No ..... Don't Know .....
B5	Do you know that cattle also have TB?	Yes ..... No ..... Don't Know .....
B6	Do you know that human can infect cattle with TB?	Yes ..... No ..... Don't Know .....
B7	Do you know that cattle can infect human with TB?	Yes ..... No ..... Don't Know .....
B8	What are the ways through which TB can be transmitted from cattle to man?	Direct contact with infected cow..... Consumption of meat ..... Milk & Milk products ..... Others (specify) .....
B9	Do you know the clinical signs of TB in cattle?	Yes ..... No ..... Don't Know .....

B10	Do you drink Milk?	Yes ..... No..... Don't Know .....
B11	How?	Sour..... Fresh..... Boiled.....
B12	Do you know why you have been told to boil milk?	Yes ..... No..... Don't Know .....
B13	Do you know that you can get TB from "Madara" or "Nono"	Yes ..... No..... Don't Know .....
B14	Have any of your cattle been coughing in the last 12 months?	Yes ..... No ..... Don't Know .....
B15	Have some of your cattle been losing weight in the last 12 months?	Yes ..... No ..... Don't Know .....

SECTION C: KNOWLEDGE OF TB

QUESTION NO.	QUESTIONS	RESPONSE OPTIONS
C1	Do you know of anybody in your family who has been coughing?	Yes ..... No ..... Don't Know .....
C2	If yes to question C1, what are the signs/symptoms you observed in the person?	Cough ..... Wasting ..... Sweat in the night ..... Body weakness ..... Others .....
C3	How many of your cattle have you sold to another herd during the last 12months?	
C4	Are there any herd around you with coughing other than" Huhu"?	Yes ..... No ..... Don't Know .....

C5	Do you think TB can be cured?	Yes ..... No ..... Don't know .....
C6	What type of treatment do you think is best for TB?	Modern medicine ..... Traditional medicine ..... Faith healing ..... Others (specify)
C7	Have you observed any member of your household with these symptoms in the past 3 months?	Cough ..... Wasting ..... Sweat in the night ..... Body weakness ..... Others .....

SECTION D: **ATTITUDE TO TB**

QUESTION NO.	QUESTIONS	RESPONSE OPTIONS
D1	Do you think diseases can be transferred from cattle to man?	Yes ..... No ..... Don't Know .....
D2	If yes, how?	.....
D3	If no, why not?	.....
D4	Do you think that it is important to protect yourself while handling diseased cattle?	Yes ..... No ..... Don't know .....
D5	Have you been given BCG day old vaccination?	Yes ..... No ..... Don't know .....
D6	What will you do with TB suspect in your herd?	Culling the animal..... Attempt to treat..... Slaughter.....

D7	What would you do at slaughter slab if your cattle have been found to have lesions compatible with TB?	Total condemnation..... Partial condemnation..... Pass for human consumption.....
D8	Do you consciously approach cases with that in mind?	Yes---------- No---------- Don't Know .....
D9	Do you carry out routine meat inspection in your abattoir?	Yes---------- No---------- Don't Know .....
D10	Have you run into TB suspect carcass?	Yes---------- No---------- Don't Know .....
D11	How did you handle the case?	Total condemnation..... Partial condemnation..... Pass for human consumption.....
D12	Did you insist on trace back?	Yes---------- No---------- Don't Know .....
D13	What measures did you take to protect the public?	Enlightenment..... Notify the authority..... Seizure of carcass.....

SECTION E: **PRACTICES ABOUT TB**

QUESTIONNAIRE NO.	QUESTIONS	RESPONSE OPTIONS
E1	What do you do to protect yourself from diseased cattle/carcass?	Immunization ..... Good hygiene practices ..... Use of herbs ..... Prayer ..... Others (specify)
E2	Have you ever been tested for TB?	Yes ..... No .....

E3	If no, why didn't you go for the test?	Am not sick ..... I cannot have TB ..... I don't know there is treatment for it .....
E4	What kind of milk do you drink?	Fresh un-boiled milk ..... Boiled milk ..... Sour milk .....
E5	What would you do if you see your cattle having TB symptoms?	I will sell it ..... I will kill and bury it ..... I will eat it personally at home ..... Others (specify)
E6	If you have TB where would you go for treatment?	Hospital..... ..... Traditional medicine ..... Faith (Prayer) healing ..... Others (specify) .....
E7	What type of hygiene practice do you observe to protect yourself from contracting TB from cattle?e.g during milking, drinking milk or direct contact	Putting on proactive material while working ..... Limiting contact with cattle/carcass ..... Washing of hands after touching live cattle or carcass ..... Others (specify) ..... None .....
E8	What other disease condition have you experienced recently?	Brucellosis(Bakkale)..... CBPP (Huhu)..... Fasciolosis (Hanta)..... Trypanosomosis (Sammore)..... Others (specify) .....

THANK YOU FOR YOUR TIME AND RESPONSE

NAME OF INTERVIEWER----------

APPENDIX V

YOBE STATE GOVERNMENT OF NIGERIA
HOSPITALS MANAGEMENT BOARD
OFFICE OF THE EXECUTIVE CHAIRMAN

Tel: 074-522790
P. M. B. 1066

Km 2, Along Gujba
Road, Damaturu.

Our Ref:- Your Ref:- Date:- 2nd Nov. 2011

The P.M.O. I/C
Gen. Hosp. Potiskum
" " Gashua
" " Damaturu
" " Geidam

ATTENTION:
H.O.D. Patthology

RE-FIELD SURVEY OF BOVINE TUBERCULOSIS IN CATTLE AND HUMAN POPULATIONS IN NORTHEASTERN STATES OF NIGERIA

Reference to the attached letter of 26th July, 2011 concerning the above subject matter, I am directed to inform you that the field survey of bovine Tuberculosis in haman cattle team is conducting field survey on the prevalence of Bovine TB in human and cattle. The survey will involve will sputum smear for AFB.

2. In view of the above therefore, you are hereby directed to gave them your maximum co-operation during the exercise.

3- As usual, it is expected that you keep record of all activities conducted during the exercise.

YAHYA ALH. IBRAHIM
FOR EXECUTIVE CHAIRMAN